Exploring the pathogenic potential of myelin-reactive Th1 and Th17 cells in central nervous system autoimmune disease

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Declaration

I declare that this thesis has been submitted by myself, describes my own work and has not been submitted in any other application for a higher degree.

The initial pMOG Th1/Th17 optimisation of the *in vitro* polarisations, and subsequent pMOG Th1/Th17 *in vivo* passive transfer experiment was performed by Dr Richard O'Connor prior to holding a Home Office license.

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Abstract

The activation of naïve T cells results in their proliferation and differentiation into a particular T-helper (Th) phenotype, namely Th1, Th2 or Th17 cells. This thesis focuses on the role of pro-inflammatory Th1 and Th17 cells in the induction of autoimmune disease of the central nervous system (CNS), using murine experimental autoimmune encephalomyelitis (EAE) as the model.

Classically, EAE has been considered to be a Th1-mediated disease. However, since the emergence of the Th17 cells, there has been a paradigm shift towards Th17 cells being the key pathogenic subset in autoimmune disease. This thesis established robust protocols for the differentiation of naïve T cells into myelin-reactive Th1 or Th17 cells, producing 'clean' populations devoid of any contaminating cells. Passive T cell-transfer experiments revealed that myelin-reactive Th1 cells could induce EAE, whereas Th17 cells could not. This lack of disease correlated with the inability of the Th17 cells to accumulate in the non-inflamed CNS. Myelin-reactive Th1 cells did have this capability and only once inflammation was established, could Th17 cells be identified in the CNS, potentially exacerbating the disease. After these differences were observed, the project investigated two main aims: 1) to identify differences in homing molecule expression on Th1 and Th17 cells which could explain the difference in their ability to home to the CNS, and to investigate the functional significance of such differences, by molecular blockade; 2) to investigate the requirements for three key cytokines in EAE pathogenesis in passive T cell transfer models, investigating IFN-γ, IL-17 and TNF-α.

P-selectin glycoprotein ligand-1 appeared to be important for the initial entry of inflammatory T cells into the CNS. Th1 cells deficient in IFN-γ were capable of inducing EAE. A proportion of the mice developed "atypical" clinical signs, which correlated with T cell infiltration predominantly of the brain, rather than the spinal cord. This atypical EAE may be IL-17 dependent. In conclusion, this thesis indicates the importance of not focusing all resources and therapeutic approaches on Th17-induced inflammation, as Th17 cells may not play such a major role as previously thought.

List of Publications

1. Immune Cell Entry to Central Nervous System – Current Understanding and Prospective Therapeutic Targets. **Catriona T. Prendergast** and Stephen M. Anderton, Endocrine, Metabolic & Immune Disorders - Drug Targets, **2009**, 9, 315-327
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List of Abbreviations

Ag	Antigen
APC	Antigen presenting cell
BBB	Blood brain barrier
C2GnT-I	Core 2 β-1.6-N-acetyl glycosaminyltransferase
CD	Cluster of differentiation
CCR-	Chemokine receptor-
CFA	Complete Freund's adjuvant
CNS	Central nervous system
CSF	Cerebrospinal fluid
CTLA-4	Cytotoxic T-lymphocyte antigen-4
DC	Dendritic cell
DN	Double negative
DP	Double positive
EAE	Experimental autoimmune encephalomyelitis
EAU	Experimental autoimmune uveitis
EBNA	EBV nuclear antigen
EBV	Epstein Barr virus
FoxP3	Forkhead BoxP3 protein
FucT-VII	α-(1,3)-fucosyltransferase-VII
GlyCAM-1	Glycosylation-dependent cell adhesion molecules-1
GM-CSF	Granulocyte macrophage colony-stimulating factor
HLA	Human leukocyte antigen
ICAM-1	Intercellular adhesion molecule-1
IFN-γ	Interferon-γ
IL-	Interleukin-
i.p.	intra-peritoneal
IP-10	IFN-γ-induced protein-10
IPEX	Immunodysregulation, polyendocrinopathy, enteropathy, and X-linked
ITAM	Immunoreceptor tyrosine-based activation motif
i.v.	intravenous
JAK	Janus Kinase
Lck	Lymphocyte-specific protein tyrosine kinase
LFA-1	Lymphocyte function-associated antigen-1
LPS	Lipopolysaccharide
MAdCAM-1	Mucosal addressin cell adhesion
MBP	Myelin basic protein
MHC	Major histocompatability
MIP-1α	Macrophage inflammatory protein-1α
MMP	Matrix metalloproteinase
MOG	Myelin oligodendrocyte glycoprotein
MRI	Magnetic resonance imaging
MS	Multiple sclerosis
NK	Natural killer
NOD	Non obese diabetes
PAMP	Pathogen associated molecular pattern

PCR	Polymerase chain reaction
PD-1	Programmed death-1
PLP	Proteolipid protein
PML	Progressive multifocal leukoencephalopathy
PPMS	Primary progressive MS
PRR	Pattern recognition receptor
PSGL-1	P selectin glycoprotein ligand-1
Ptx	Pertussis toxin
RA	Rheumatoid arthritis
RAG	Recombinase activating gene
ROR-	Retinoic acid-related orphan receptor-
RRMS	Relapsing remitting MS
RT-qPCR	Reverse transciptase quantitative PCR
s.c.	sub-cutaneous
SOCS	Suppressor of cytokine signalling
SPMS	Secondary progressive MS
STAT	Signalling transducer and activator of transcription
T-bet	T box expressed in T cells
TCR	T cell receptor
TGF-β	Transforming growth factor-β
TLR	Toll-like receptor
Th	T helper
TNF	Tumour necrosis factor
Treg	T regulatory cell
VCAM-1	Vascular cell adhesion molecule-1
#	denotes ‘number’

1 Introduction

1.1 Context

The immune system has evolved to protect the body against invading pathogens through both the innate and adaptive systems, with the innate system initiating the induction of the adaptive immune system. Naïve $CD4^+$ T cells are activated in the peripheral lymph nodes by recognition of their cognate peptide-MHC class II complex displayed on the surface of antigen presenting cells (APCs), leading to their proliferation and differentiation into various T helper (Th) cell phenotypes, determined by the cytokine milieu in the immediate environment. This allows the T effector cells to orchestrate a qualitatively appropriate immune response. Importantly, the immune system must also be able to discriminate between self and non-self to avoid autoimmune pathology. This is avoided through central and peripheral tolerance mechanisms. Autoimmune disease arises when both of these mechanisms fail, and a destructive immune response is mounted against self antigens, resulting in the destruction of self tissue and organs. Multiple sclerosis (MS) is an autoimmune disease of the central nervous system (CNS) (Hafler et al., 2005). It is thought to be mediated by pro-inflammatory $CD4^+$ T cells, namely Th1 and Th17 cells. In this thesis, the pathogenic ability of Th1 and Th17 cells is investigated through the use of the murine model of MS, experimental autoimmune encephalomyelitis (EAE). This model recapitulates the main features of multiple sclerosis and allows the study of the roles of the various T effector cell lineages, to assess their contribution to disease pathogenesis.

1.1.1 Basic T cell biology

All lymphocytes originate from the same common lymphoid progenitor cells, haematopoietic stem cells, in the bone marrow. Whereas B cells develop within the bone marrow, a proportion of the haematopoietic stem cells migrate from the bone marrow to the thymus where they undergo several development steps to mature into T cells. The absence of a thymus, due to a genetic defect or neonatal thymectomy results in partial immunodeficiency due to a low peripheral T cell compartment

(Miller, 1961), demonstrating the importance of the thymus in T cell development. In humans, the thymus is fully developed at birth but is thought to undergo involution from adolescence onwards (Aspinall and Andrew, 2000), resulting in decreased output of newly generated T cells.

1.1.1.1 T cell development in the thymus

Two functionally distinct types of T cells are generated in the thymus: the αβ T cells and the γδ T cells, distinguished by their expression of an αβ or a γδ TCR (reviewed in (Ciofani and Zuniga-Pflucker, 2010)). When entering the thymus, the haematopoietic stem cells lack expression of the surface molecules characteristic of mature T cells. There are several stages of thymocyte development, the earliest being the 'double negative' (DN) stage in which the thymocytes lack the co-receptors CD4 or CD8 (as well as T cell specific CD3 and the T cell receptor (TCR)) (Figure 1.1). The double negative cells are extremely heterogeneous and can be subdivided into four different stages DN1, DN2, DN3a and DN3b defined on their expression of CD44, CD25 and CD117 (Porritt et al., 2004). Between the DN1 and DN2 stages gene rearrangement begins at the β-, γ- and δ-TCR gene loci and commitment towards either the αβ or γδ T cell lineage is made by DN3 (Taghon et al., 2006). The cells that have successfully rearranged γδ TCRs go on to develop into mature γδ T cells. TCR signalling and notch signalling are considered important for the decision of αβ or γδ T cell commitment (reviewed in (Kreslavsky et al., 2010)).

As this thesis investigates αβ T cells, a brief over-view of their thymic development follows. Successful rearrangement of the VDJ gene segments of the β-chain locus of the TCR, results in commitment towards the αβ T cell lineage. Those cells that do not achieve successful β-chain rearrangement undergo apoptosis. The β-chain then associates with a pTα and this heterodimer (forming the pre-TCR) complexes with the CD3 chains and is expressed on the surface of the thymocyte. This signals for the β-chain rearrangement to stop, the cells proliferate and the CD4 and CD8 molecules are expressed forming $CD4^+CD8^+$ 'double positive' (DP) thymocytes. The α-chain gene rearrangement then begins and continues until a successful α-chain is produced, and this pairs with the β-chain to form a mature αβ TCR (Zuniga-Pflucker, 2004;

Ciofani and Zuniga-Pflucker, 2010). αβ T cells are 'MHC restricted,' as they only recognise antigen when it is presented by MHC molecules on APCs. This MHC restriction is thought to be coded by the germline-encoded complementary-determining regions 1 and 2 (CDR1 and CDR2) of the TCR. These recognise the conserved feature of the MHC helices, and the peptide specificity is dictated by the highly variable CDR3s (Chothia, Boswell and Lesk, 1988; Davis and Bjorkman, 1988) (reviewed in (Mazza and Malissen, 2007). Numerous different cell types act as APCs within the thymus, including cortical thymic epithelial cells, medullary thymic epithelial cells and dendritic cells (DCs). These APCs and the thymic structure itself have an important role in thymic selection which is explained below.

1.1.1.2 Positive Selection

The DP thymocytes undergo positive selection based on the affinity of their αβ TCR for MHC-peptide complexes on thymic epithelial cells. This is when DP thymocytes that have no affinity for the self-peptide-MHC class I and class II complexes (presented by the DCs) are deleted. This results in positive selection for those DP thymocytes with functional TCRs with sufficient avidity for self-peptide-MHC-complexes. These positively selected cells are then induced to differentiate into mature T cells and lose expression of either CD4 or CD8, resulting in the formation of $CD4^+$ or $CD8^+$ single-positive thymocytes. Those selected on MHC class I molecules become $CD8^+$ cells by downregulating CD4, and those selected on MHC class II molecules become $CD4^+$ cells by downregulation of CD8 (Zuniga-Pflucker, 2004).

1.1.1.3 Negative Selection

The thymocytes move from the cortex of the thymus towards the medulla region. Here they undergo negative selection, also known as clonal deletion. This is the elimination of both DP ($CD4^+CD8^+$) as well as single-positive ($CD4^+CD8^-$ or $CD4^-CD8^+$) thymocytes that respond strongly to self peptide. Many studies have shown that strength of signal of the TCR for the peptide-MHC complex determines the fate of thymocytes during the positive and negative selection process (Ashton-Rickardt et

al., 1994; Sebzda et al., 1994; Germain, 2001; Daniels et al., 2006). The elimination of strongly self-reactive T cells from the thymus is known as central tolerance and is vital for the prevention of autoimmune disease.

1.1.1.4 Treg development in the thymus

$CD4^+$ natural T regulatory cells (nTreg), which are vital for the maintenance of peripheral tolerance and the suppression of inflammation, have been shown to develop within the thymus as well. Forkhead Box P3 protein (FoxP3) was identified as a marker of Treg (Hori, Nomura and Sakaguchi, 2003). nTreg are now known to be produced late in thymic T cell development, and are differentiated from single-positive CD4 T cells, to produce $FoxP3^+$ Treg, which then migrate out in to the periphery (Fontenot et al., 2005). In polyclonal mice, conventional T cells and nTreg develop at a constant ratio, however in TCR transgenic systems that lack the RAG genes i.e. the endogenously rearranged TCR chains, nTreg cells are absent (Lafaille et al 1994; Picca et al 2006). An increase in development of nTreg was observed if the cognate ligand for the respective TCR transgenic was expressed within the thymus (Jordan et al, 2001; Kawahata et al, 2002). Processes distinct from either positive or negative selection allow for the development of nTreg in the thymus. This allows for Treg to exit the thymus into the periphery and to respond to specific self-peptides with high affinity. The exact mechanisms involved in their own selection in the thymus are still being determined however numerous factors including the endogenous TCR gene rearrangement, the affinity for self-peptide, TGF-β signalling as well as the organisation of the thymic structure are important (Kawahata et al, 2002; Relland et al, 2009).

A two-step model of nTreg development in the thymus has recently been proposed. Firstly, TCR signalling results in the upregulation of the IL-2Rα (CD25) and this directly leads to the induction of FoxP3 expression (Lio and Hsieh, 2008). Transforming growth factor (TGF)-β is also involved in the development of nTreg. Mice deficient in TGF-βR I specifically within the T cells, had reduced numbers of $CD4^+$ $CD25^+$ $FoxP3^+$ thymocytes in young mice, however this reduction in number was only temporary (Liu et al., 2008b). If both TGF and IL-2 signalling was lost,

$CD4^+$ $CD25^+$ $FoxP3^+$ thymocyte numbers were permanently reduced (Liu et al., 2008b). Co-stimulation via CD28 has also been shown to upregulate FoxP3 expression in developing thymocytes independent of IL-2 (Tai et al., 2005). Once developed, mature $CD4^+$ and $CD8^+$ single positive T cells and $FoxP3^+$ nTreg are exported out of the thymus and enter the peripheral T cell repertoire.

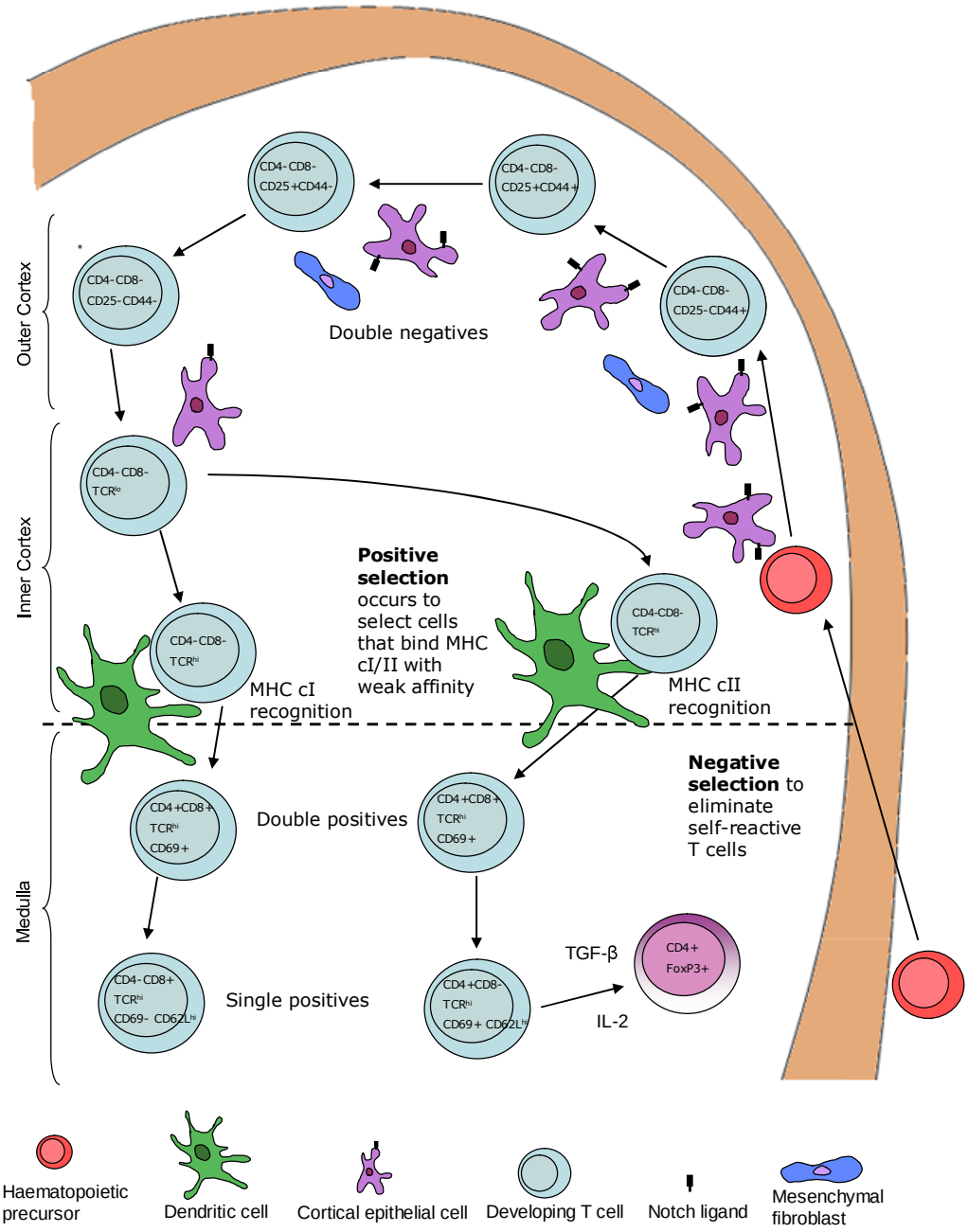

Figure 1.1 Over-view of αβ-T cell development within the thymus.

Haematopoietic stem cells enter the thymus and develop into double negative precursors through Notch ligand signalling. Thymocyte differentiation is characterised by the expression of well defined cell surface markers, for example those listed in the diagram, CD4, CD8, CD44 and CD25. Notch signalling induces a complex programme of T cells maturation in the thymus resulting in the development of single positive $CD4^+$ or $CD8^+$ T cells that migrate out into the peripheral pool of naïve T cells. Positive and negative selection in the thymus ensures the maturation of functional T cells and minimises the maturation of self-reactive T cells. $CD4^+$ cells also develop into $FoxP3^+$ Treg in the thymus. Adapted from Zúñiga-Pflücker Nature Reviews Immunology (Zuniga-Pflucker, 2004).

1.1.2 Development of immune response to infection

The innate immune system is vital for the early recognition of infection and the triggering of the proinflammatory response to invading pathogens (Medzhitov and Janeway, 2000). In contrast the adaptive immune system is responsible for the elimination of specific pathogens later during infection and, importantly, for the generation of immunological memory.

1.1.2.1 The innate immune system

Cells of the innate immune system include mast cells, eosinophils and basophils, and the phagocytic cells macrophages, neutrophils and dendritic cells. These are all derived from common myeloid progenitor cells within the bone marrow. Mast cells, basophils and eosinophils are granular cells that function by release of proinflammatory agents, for example histamine by mast cells and basophils, and cationic proteins and cytokines by eosinophils (reviewed in (Abraham and St John, 2010; Stone, Prussin and Metcalfe, 2010). These cells have important roles in host defence and allergy. Mast cells reside in tissues whereas basophils circulate in the blood. Basophils have also been suggested to act as APCs and to be important for modulating Th2 inflammation (Min et al., 2004; Oh et al., 2007; Schroeder, 2009) although they have recently been shown not to be required for Th2 responses in helminth infection and allergy (Hammad et al., 2010; Phythian-Adams et al., 2010).

Other important mediators of the innate immune system are the phagocytic cells, macrophages, neutrophils and DCs. These are able to phagocytose and kill invading pathogens. This is particularly true if opsonized by antibody which facilitates antibody-Fc receptor binding on the phagocyte and complement (reviewed in (Carroll, 2004)). Complement mediates both humoral and cellular interactions within the immune response including phagocytosis and it can be activated via three different routes, the classical and alternative pathways and the lectin pathway.

Both macrophages and DCs are also an important link between the innate and adaptive immune systems, as they act as APCs to naïve T cells. The APCs are able to

phagocytose the foreign invading antigens, process the antigen internally and present resulting peptide fragments on their surface within the binding cleft of MHC molecules. The activation of naïve T cells by antigen presentation signals the activation of the adaptive immune response. The adaptive immune response is required for key reasons: 1) it provides a specific response against a particular antigen or pathogen; 2) it provides a long lasting response through immunological memory, the generation of memory cells specific for a particular antigen.

1.1.2.2 The adaptive immune response

Naïve T cells emigrate from the thymus to establish the peripheral T cell pool in the lymphoid organs and blood. T cells leave the blood circulation and enter the lymph nodes via the high endothelial venules. Within the lymph node the T cells accumulate in the T-cell zone where they encounter their cognate antigen during antigen presentation by the APCs. The majority of the T cells are not activated, and re-enter circulation via the medulla and the efferent lymph into the thoracic duct (Westermann et al., 2001).

1.1.2.3 T cell activation

Bretscher and Cohn originally suggested that lymphocytes require two signals to mount an effective immune response against an antigen (Bretscher and Cohn, 1970). Over the years, this hypothesis has been refined, and it is now known that three distinct signals are required for the full activation of the adaptive immune response, or the activation of a naïve T cells: 1) activation through the TCR which recognises the peptide-MHC complex on the surface of the APC; 2) non-specific signalling through co-stimulatory molecules on the surface of the T cell and their respective ligands on the surface of the APC; and 3) cytokines produced by either the APC or the activated T cell itself induce cytokine receptor signalling within the T cell which both amplifies the activation and aids differentiation of the T cell as summarised in Figure 1.2. However, before these three signals can be elicited for T cell activation, ‘signal 0’ first has to occur. This is the signal required for DC activation to initiate antigen presentation.

1.1.2.4 Signal 0: DC activation

Immature DCs recognise pathogen-associated molecular patterns (PAMPs) (Janeway, 1989) of invading pathogens and this triggers the activation of the DC. PAMPs are small molecular motifs present on entire classes of pathogens including bacteria, viruses, fungi or parasites. PAMPs are recognised by pattern-recognition receptors (PRRs) expressed by DCs. There are numerous different PRRs, for example membrane bound or intracellular toll like receptors (TLRs) which are the most widely characterised, and intracellular cytosolic PRRs, retinoid acid-inducible gene I (RIG-I)-like receptors (RLRs) and nucleotide-binding oligomerisation domain (NOD)-like receptors (NLRs) (reviewed in (Mogensen, 2009)). Of the cytosolic PRRs, NLRs are implicated in recognising bacterial components (Inohara et al., 2005) whereas RLRs are able to recognise viral dsRNA (Yoneyama et al., 2004). LPS is the prototypical PAMP and signals through TLR4 (Kim et al., 2007). TLR9 is able to recognise bacterial and viral DNA (Hemmi et al., 2000; Hochrein et al., 2004).

The effect of PRR ligation is phagocytosis, upregulation of MHC, costimulatory molecules and cytokine production. Following the antigen uptake by DCs as mediated by PRR signalling, the antigen is processed internally and is presented on the surface of the DC on the MHC molecules. The DCs migrate to the regional lymph nodes to present their antigenic peptides to the naive T cells. Signal 0 therefore provides the important link between the innate and adaptive immune systems.

1.1.2.5 Signal 1: Activation through the TCR

The TCRα:TCRβ heterodimer forms the binding site for the cognate peptide-MHC complex. Adjacent to these is the CD3 complex, which consists of one δ, one γ, two ε subunits and one intracellular ζ homodimer. The CD3 chains have extracellular domains and cytoplasmic tails. The cytoplasmic tails of the CD3 chains contain immunoreceptor tyrosine-based activation motifs (ITAMs) which assist in the signal transduction after their phosphorylation. Associated with the TCR are the co-receptors CD4 or CD8. Lymphocyte-specific protein tyrosine kinase (Lck), a

member of the Src family of tyrosine kinases is associated with the cytoplasmic tail of the CD4 or CD8 co-receptors (reviewed in (Nakayama and Yamashita, 2010)).

Following TCR ligation, the CD4 or CD8 co-receptors cluster with the TCR, allowing activated Lck to phosphorylate the tyrosine residues on the ITAMs on the CD3 and ζ chains. This phosphorylation leads to ZAP-70 (a tyrosine kinase of the spleen tyrosine kinase (Syk) family) binding to the ITAMs bringing it into the close vicinity of Lck and allowing Lck to phosphorylate ZAP-70. Phosphorylated and activated ZAP-70 initiates a series of complex intracellular signalling cascades through the phosphorylation of LAT molecules. This leads to activation of various signalling pathways including a) the Ras/ERK MAPK cascade, b) the Ca^{2+}/calcineurin/NFAT pathway as well as c) the PKC/NFκB pathway (reviewed in (Nakayama and Yamashita, 2010) which lead to activation of the T cell and gene transcription.

1.1.2.6 Signal 2: Co-stimulation

If a T cell receives signalling through the TCR only, without any co-stimulation, this results in the T cell entering an anergic state in which it is non-responsive to further TCR stimulation, even in the presence of costimulation. Only through co-stimulatory signalling can anergy be avoided and T cell activation achieved (reviewed in (Schwartz, 2003). CD28, which is constitutively expressed on the surface of the T cell, is the key co-stimulatory molecule providing initial robust signalling and is important for T cell proliferation, cytokine production, cell survival and cellular metabolism, and therefore, anergy prevention (Green et al., 1994; Boise et al., 1995). This is highlighted as CD28-deficient mice have severely impaired CD4 T cell proliferation (Green et al., 1994). CD28 binding to its ligands, CD80 (B7-1) and CD86 (B7-2) on the surface of the APC, leads to a series of T cell signalling pathways and to upregulation of many genes involved in T cell activation. Antibodies against CD80 and CD86 can inhibit T cell activation *in vitro*, highlighting the importance of CD28 co-stimulation for T cell activation (Salomon and Bluestone, 2001). Importantly, signalling through the TCR and CD28 results in IL-2 production, whereas anergic cells produce little IL-2 (reviewed in (Powell et al., 1998)). The

activated APCs therefore provide signal 1 through the MHC/peptide complex and the signal 2 through co-stimulation. Blocking the CD28/B7 interaction in T cell clones leads to T cell anergy (Tan et al., 1993).

Numerous other molecules on the surface of T cells have been shown to have co-stimulatory properties including CD5, OX40 and inducible co-stimulator (ICOS). There are also co-inhibitory molecules, which are upregulated after CD28 engagement, for example, cytotoxic T-lymphocyte antigen-4 (CTLA-4), programmed death-1 (PD-1) and B and T lymphocyte attenuator-4 (BTLA-4) (reviewed in (Salomon and Bluestone, 2001) and (Greenwald, Freeman and Sharpe, 2005)). Ligation of CTLA-4, which competes for the same ligands as CD28, results in the inhibition of T cell activation (Walunas, Bakker and Bluestone, 1996; Salomon et al., 2001).

CTLA-4-deficient mice develop lymphoproliferative disease, adding support to its role in inhibiting T cell activation and proliferation (Tivol et al., 1995; Waterhouse et al., 1995). More recently, Treg have been shown to require CTLA-4 for their suppressive function (Tang et al., 2004). Although also a negative regulator of T cell responses, PD-1 has a very distinct regulatory pathway from CTLA-4, due to the different expression patterns of their ligands. PD-1 has two ligands, programmed death ligand-1 (PD-L1) and PD-L2 (Freeman et al., 2000; Latchman et al., 2001). PD-L1 is expressed on leukocytes and in non-lymphoid tissues whereas PD-L2 is expressed on DCs and monocytes only (Freeman et al., 2000; Yamazaki et al., 2002). Blockade of PD-1 has been shown to accelerate autoimmune disease in non obese diabetic (NOD) mice and EAE (Ansari et al., 2003; Salama et al., 2003). CTLA-4-deficient mice develop autoimmune disease or lymphoproliferative disease early in life in comparison to PD-1-deficient mice, which develop autoimmunity after several months (Waterhouse et al., 1995; Okazaki and Honjo, 2006). This supports the hypothesis that CTLA-4 is required early in the initiation of a T cell response within the lymph node. CTLA-4 and PD-1 have distinct roles in controlling autoreactive T cells that have escaped central tolerance in the thymus.

1.1.2.7 Signal 3: Cytokines

The third signal required for full activation and differentiation of a naïve T cell is elicited through the cytokines produced by the APC and by the T cell itself. Without the third signal from inflammatory cytokines, $CD8^+$ T cells fail to develop into optimal cytotoxic T cells (reviewed in (Mescher et al., 2006)). IL-12 and IFN-α/β are important signal 3 mediators for $CD8^+$ T cells leading to full activation of the cells, after TCR/MHC class I and co-stimulation signalling resulting in effector function as well as memory (Mescher et al., 2006). TNF-α, and IL-1 have been found to be important pro-inflammatory cytokines in the activation of $CD4^+$ T cells (Pape et al., 1997; Ben-Sasson et al., 2009) although their exact role and mechanism of action is still to be elucidated.

The cytokine milieu when a CD4 T cell is activated is vital for the differentiation of the naïve T $CD4^+$ T cell into a particular Th phenotype. The cytokine signalling results in down-stream activation of specific signal transducer and activator of transcription (STAT) proteins which results in differentiation down a specific route. $CD4^+$ T cells can differentiate into at least four known subsets: Th1, Th2, Th17 and adaptive (a)Treg cells (Mosmann et al., 1986; Bettelli, Korn and Kuchroo, 2007; Curotto de Lafaille and Lafaille, 2009). Th1 cells require cytokine signalling through interferon (IFN)-γ and IL-12 to activate downstream STAT4 and STAT1 signalling (Seder et al., 1993). Th2 cells require signalling through IL-4 to activate STAT5 and STAT6 signalling (Le Gros et al., 1990). Th17 cells are differentiated in the presence of IL-6 and TGF-β (Veldhoen et al., 2006) via STAT3 signalling and iTregs in the presence of TGF-β and IL-2 (Chen et al., 2003; Yu and Malek, 2006) (reviewed in (Zhu and Paul, 2010). The details of $CD4^+$ T cell lineages and their differentiation and function is given in Section 1.2. Signalling through signals 1 and 2 result in the chromatin remodelling for gene activation, whereas signalling through signal 3 is important for the specific cytokine receptors that results in activation of lineage specific genes.

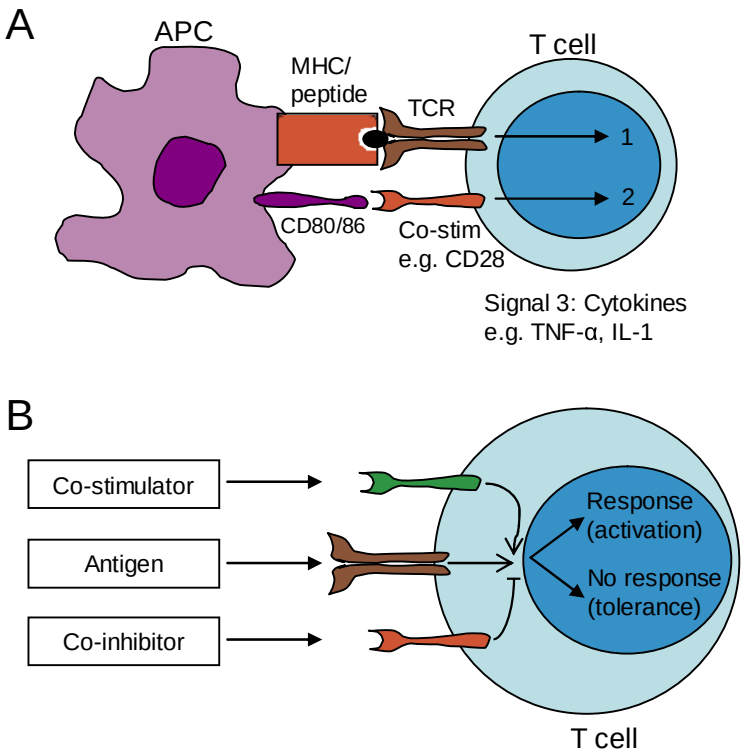

Figure 1.2 Signals required for full T cell activation.

A, $CD4^+$ T cells require three signals for successful activation: 1) signalling with the MHC cII/peptide complex on the APC through the T cell receptor; 2) signalling through co-stimulatory molecules, for example CD28 on the T cell surface with its ligands CD80 or CD86 on the APC surface; 3) signalling through cytokines produced by the surrounding APCs or the T cell itself, e.g. TNF-α and IL-1; Signalling through signal one only, i.e. the TCR, in the absence of co-stimulation results in no response and the induction of tolerance or anergy. Conversely, in the absence of signal one, ligation of co-stimulatory receptors does not induce a T cell response. **B,** The role of co-stimulatory molecules and co-inhibitory molecules in the regulation of T cell receptor signals. Although essential for a T cell response, signalling through the TCR does not automatically determine activation or inhibition, and depends on signals from co-stimulatory and co-inhibitory molecules to determine T cell activation, or T cell inhibition. A balance between these two opposing signals determines the outcome. Adapted from Chen, L, 2004, Nature Reviews Immunology (Chen, 2004).

The major advantage of the adaptive immune system is the generation of long-term immunological memory to antigen. Antigen recognition is determined by TCRs and BCRs which show a huge ability for diversity through recombination and (in the case of the BCR) somatic hypermutation. This great diversity provides the opportunity for the generation of self antigen-reactive receptors and hence the risk of an autoimmune response. Central tolerance mechanisms have been discussed. Because these are not

100% effective, autoreactive T cells do escape into the T cell repertoire in the periphery and the peripheral tolerance mechanisms are in place to control these cells (reviewed in (Anderton and Wraith, 2002). However, peripheral tolerance can also be broken with the ultimate development of clinical autoimmune disease.

1.1.3 Autoimmune disease: Multiple Sclerosis

MS is a complex disease of the CNS that is thought to be initiated by autoimmune inflammation. The prevalence of the disease varies in different areas in the world and is found to be highest in northern Europe, southern Australia and North America (Kurtzke, 1991). The reason for this is not understood and the etiology of MS is still unknown. There is a strong genetic component, but several environmental factors have been proposed (reviewed in (Noseworthy et al., 2000).

There are four pathological sub-types of demyelination in MS, as described by Lassmann and Lucchinetti:
Pattern I) active demyelination with T cell and macrophage dominated inflammation. Lesions are centred around the blood vessels.
Pattern II) active demyelination with T cell and macrophage dominated inflammation as well as deposition of IgG and complement antigen. The lesions are also centred around blood vessels.
Pattern III) inflammatory infiltrate made up of T cells, macrophages and microglia with no Ig or signs of complement. These exhibit specific loss of myelin protein myelin associated glycoprotein (MAG) and not of any other myelin proteins.
Pattern IV) T cell and macrophage dominated inflammation, with no Ig deposition or complement. The demyelination here is associated with oligodendrocyte death (Lucchinetti et al., 2000).

Patterns I and II clearly show signs of autoimmune encephalomyelitis and in contrast, patterns III and IV exhibit oligodendrocyte dystrophy (Lucchinetti et al., 2000) highlighting the clear heterogeneity in demyelination of MS lesions which is reflected in the heterogeneity of disease from patient to patient. However, an MS

patient can present all the patterns of demyelination and the pattern is not predictive of MS clinical course.

1.1.3.1 Symptoms of MS

Disease often starts with sensory disturbances, optic neuritis, clumsiness and limb weakness. As disease progresses this develops into cognitive impairment, vertigo, progressive paralysis in all four limbs, spasticity, ataxia and apraxia. Diagnosis is made on clinical manifestations as well as a defined set of laboratory criteria (Poser et al., 1983) and more recently magnetic resonance imaging (MRI) (Miller et al., 1998b) as well as the detection of B-cell-produced oligoclonal immunoglobulin in the cerebrospinal fluid (CSF) (reviewed in Noseworthy et al 2000 (Noseworthy et al., 2000).

1.1.3.2 Clinical courses of MS

A relapsing-remitting course (RRMS) develops in approximately 80% of MS patients with a 2:1 incidence ratio in females:males (reviewed in Noseworthy et al, 2000; Compston and Coles 2008 (Noseworthy et al., 2000; Compston and Coles, 2008). RRMS is characterised by periods of symptoms of disease followed by months to years of remission. This form of MS usually starts with a clinically isolated syndrome (CIS; for example, optic neuritis or evidence of brain stem dysfunction) and the majority of patients that experience this later develop MS. Secondary progressive MS (SPMS) eventually develops in the majority of patients with RRMS, and indicates a progressive neurologic decline without any periods of remission. Primary progressive MS (PPMS) affects ~20% of MS patients (Fisniku et al., 2008). It is characterised by a gradually progressive clinical course from onset, with no periods of remission and has similar incidence between males and females (Fisniku et al., 2008). The clinical courses of MS are summarised in Figure 1.3.

Importantly, there are forms of ‘borderline’ MS diseases including Marburg’s disease (also known as fulminant MS), neuromyelitis optica (NMO; also known as Devic’s disease), and Balo concentric sclerosis which can often be confused with MS.

Marburg type MS has a very aggressive clinical course with large demyelinating plaques in the hemispheric white matter of the brain. The disease evolves rapidly and can result in death after only a few months (reviewed in (Capello and Mancardi, 2004). Balo concentric sclerosis is characterised by lesions in the brain in the shape of concentric rings and appears to be more common in populations of Oriental origin (Capello and Mancardi, 2004). NMO is more common in Asiatic people and usually initiates with CIS i.e. optic neuritis. From this patients develop inflammation of the spinal cord, myelitis. After these first two defining events, the disease can be monophasic or relapsing (Bergamaschi and Ghezzi, 2004; Lennon et al., 2004). Importantly the autoantigen for NMO has been identified as aquaporin 4 (Lennon et al., 2005). These associated disorders are now clinically distinguishable from MS once disease is established.

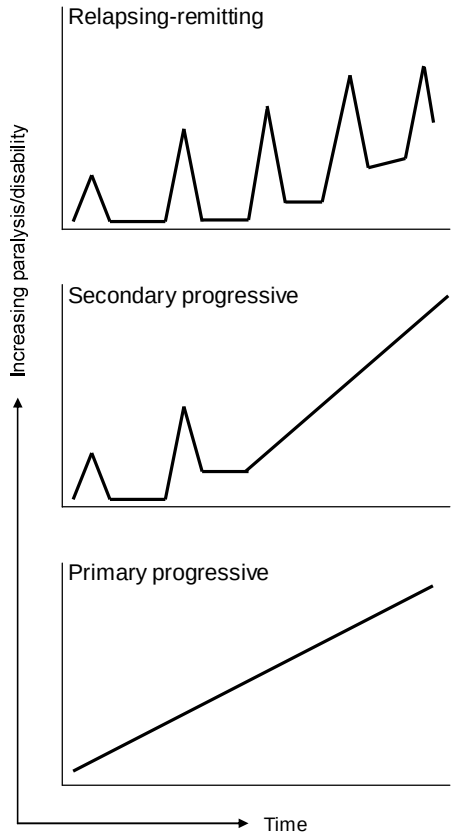

Figure 1.3 Clinical courses of different sub-types of multiple sclerosis.

MS manifests itself in several different forms in different patients, ranging from relapsing remitting disease (top), secondary progressive disease which normally develops from relapsing remitting MS (middle) and primary progressive MS (bottom).

1.1.3.3 The genetics of MS

MS has a strong genetic component. Studies in twins and siblings have shown that there is a higher risk of developing MS in monozygotic twins (25-30% concordance), dizygotic twins and non-twin siblings (2-5% concordance), as compared to unrelated adoptees from non-affected parents (<0.2% concordance). This low risk in unrelated adoptees reflects the risk in the general population (Willer et al., 2003; Compston and Coles, 2008). In the 1970s the first association between MS and the alleles of the MHC was identified (Compston, Batchelor and McDonald, 1976; Terasaki et al., 1976). These have since been specified to human leukocyte antigen (HLA) serotypes DR15 and DQ6 and the corresponding genotypes: DRB1*1501, DRB5*0101, DQA1*0102 and DQB2*0602 (Olerup and Hillert, 1991). These are all associated with the MHC and therefore have a role in T cell activation, supporting the thought that MS is T cell mediated.

In a large genomewide association study, another set of single nucleotide polymorphisms (SNPs) associated with MS was recently identified, outside of the MHC region. These SNPs are within the IL-2Rα and IL-7Rα chains (Hafler et al., 2007). Polymorphisms within the IL-2Rα (CD25) supports the idea that regulation of the immune response is required during MS and correlating with this, Treg have been shown to have impaired function in MS (Viglietta et al., 2004; Haas et al., 2005). IL-7 signalling may be needed for the generation of autoreactive T cells in MS patients (Bielekova et al., 1999).

Importantly, genetic studies have also brought to light the protective effects of some alleles, namely HLA-C5 (Yeo et al., 2007), HLA-DRB1*11 (Ramagopalan et al., 2007; Dean et al., 2008) and HLA-B*44 (Silva et al., 2009; Healy et al., 2010).

The association of the key risk alleles of MS to the immune system add weight to the thought of the disease being autoimmune mediated. Also, the association of MS with the MHC class II molecule partly led to the hypothesis that MS was a $CD4^+$ T cell mediated disease.

1.1.3.4 Environmental factors contributing to MS

There are a few environmental factors that are implicated to have an important role in the pathogenesis of MS. The global distribution of MS can be generalised as increasing with distance north or south of the equator. Also, the risk of developing MS has been correlated to the place of residence during childhood. People have decreased risk if they migrate from high-risk regions to low-risk regions during childhood, or have an increased risk if they migrate from low-risk to high-risk regions in childhood (before the age of 15), as compared to the population in their place of origin (Dean and Kurtzke, 1971; Elian, Nightingale and Dean, 1990). The contributing factors of vitamin D and viral infection in MS will now be discussed.

1.1.3.4.1 Vitamin D

The general observation of increased incidence of MS with increasing distance from the equator has been attributed to vitamin D levels. People who, during their childhood, spent large amounts of time outdoors in the sunlight have been found to have a much lower risk of developing MS (van der Mei et al., 2003; Kampman, Wilsgaard and Mellgren, 2007) and this was also observed between pairs of monozygotic twins (Islam et al., 2007). Also, the intake of vitamin D through supplements is thought to decrease the risk of developing MS (Munger et al., 2004). Serum levels of vitamin D have been observed to be significantly lower in MS patients as opposed to healthy controls (Soilu-Hanninen et al., 2005; Correale, Ysrraelit and Gaitan, 2009), however this was not observed in all studies (Barnes et al., 2007; Kragt et al., 2009).

The exact mechanisms by which vitamin D may be having a beneficial effect in MS are still being elucidated. However it has been found that Vitamin D (or 1,25-dihydroxyvitamin D(3) as the active form) inhibits the production of pro-inflammatory cytokines by *in vitro* cultured human $CD4^+$ $CD25^-$ effector T cells, and in contrast promoted the development of $CTLA4^+$ $FoxP3^+$ Treg resulting in an anti-inflammatory effect (Jeffery et al., 2009). Correlating with this, several studies have looked at the effect of vitamin D3 on MS patients with results implicating a role in inducing a regulatory phenotype. One study showed that proliferation of $CD4^+$ T

cells was inhibited by vitamin D3 and that the cells adopted a more regulatory phenotype by inducing an increased development of IL-10-producing cells (Correale, Ysrraelit and Gaitan, 2009). A second study observed increased serum levels of TGF-β1 in patients supplemented with vitamin D3 for six months (Mahon et al., 2003).

Numerous studies have indicated that exogenous vitamin D is protective in the pathogenesis of EAE (Lemire and Archer, 1991; Spach et al., 2006; Pedersen et al., 2007; Chang et al., 2010) although varied mechanisms of action were proposed. These include inhibition via vitamin D mediated IL-10-production and suppression (Spach et al., 2006), inhibition of chemokine-mediated monocytes trafficking (Pedersen et al., 2007), or inhibition of pro-inflammatory Th17 differentiation (Chang et al., 2010). Vitamin D also been shown to induce tolerogenic DCs which can then promote $FoxP3^+$ Treg and these mediate autoimmune responses (as reviewed in (Adorini and Penna, 2009).

1.1.3.4.2 Viral infection

Epidemiological data and the inflammatory nature of the lesions in MS patients have led to the consideration of MS being driven by infection. Another explanation for the change in risk of MS after migration at an early age could be early exposure to infection. Several viral infections have been associated with pathogenesis of MS, including Epstein-Barr virus (EBV), and measles and rubella (Compston and Coles, 2008). The majority of humans are infected with EBV and it establishes a lifelong dormancy within the memory B cell pool, with occasional reactivation and therefore continual viral production. MS patients exhibit higher prevalence of anti-EBV antibodies, indicating prior infection with EBV compared to healthy controls (Ascherio and Munch, 2000; Pohl et al., 2006; Banwell et al., 2007). Interestingly, increased levels of IgG against the EBV nuclear antigen (EBNA) in normal people is associated with the increased risk of developing MS later in life as compared to healthy controls (Sundstrom et al., 2004; Levin et al., 2005;

DeLorenze et al., 2006). This was demonstrated in several short term and long term studies with MS patients. However, despite these apparent correlations, the mechanism by which EBV may contribute to MS pathology is still unclear. A recent study investigating several different viral agents to determine if they act as viral triggers to MS found that CIS patients exhibited elevated humoral and cellular immune responses to EBNA-1, but not to other viral agents, i.e. human cytomegalovirus (HCMV) or measles (Lunemann et al., 2010), and this appeared to correlate with progression to MS. The study suggested EBNA1-specific IgG titers could be used as a prognostic marker for disease progression to MS in CIS patients.

1.1.3.5 Treatments of MS

There are various different forms of treatment available for MS, as outlined below.

1.1.3.5.1 Interferon-β (IFN-β-1b and IFN-β-1a; Avonex®, Rebif®, Betaseron®)

Interferon-β has become one of the first-line agents used for the treatment of RRMS having exhibited good efficacy in several phase III trials (IFNB-MS-Study, 1993; IFNB-MS-Study, 1995; Durelli et al., 2002). Treatment with IFN-β resulted in the reduction in the rate of clinical relapse and in the development of new lesions as determined by MRI. Despite its known efficacy, the exact mechanism by which IFN-β elicits its beneficial effects is still a matter of debate. It is thought to generally shift the pro-inflammatory cytokine production, for example in a Th1 response, towards a more anti-inflammatory/regulatory cytokine environment (Ozenci et al., 2000a; Ozenci et al., 2000b; Zang et al., 2003). Only two thirds of MS patients respond to IFN-β treatment (Arnason, 1999; Chiu et al., 2009). This has recently been attributed to the ability of IFN-β to inhibit Th1 responses, by inducing increased IL-10 production, but not Th17-mediated responses as shown in EAE (Axtell et al., 2010). Correlating with this, RRMS patients that were non-responders to IFN-β treatment were found to have higher IL-17 levels (Axtell et al., 2010).

1.1.3.5.2 Glatiramer acetate (Copaxone®)

This is a synthetic amino acid polymer composed of tyrosine, glutamate, alanine and lysine and is used in the treatment of RRMS. It results in the reduction in the rate of clinical relapse and a slight reduction in the development of new lesions (Johnson et al., 1995; Mancardi et al., 1998; Comi, Filippi and Wolinsky, 2001). It is thought to shift the T cell response from a pro-inflammatory Th1 response towards an anti-inflammatory/regulatory Th2 response (Miller et al., 1998a; Duda et al., 2000). In EAE, glatiramer acetate has recently been shown to downregulate Th17-associated pro-inflammatory cytokines IL-6, IL-23, TNF-α and IL-17 and this may have an indication of the mechanism of action leading to its disease suppressive activity observed in MS patients (Begum-Haque et al., 2008).

1.1.3.5.3 Mitoxantrone (Novantrone®)

Mitoxantrone is an immunosuppressive cytotoxic agent. It intercalates with DNA causing single-stranded and double-stranded breaks to occur and also inhibits DNA repair, thus able to suppress an inflammatory response from proliferating cells namely B and T cells and macrophages (Fidler, DeJoy and Gibbons, 1986; Fidler et al., 1986; Rosenberg, Carvlin and Krugh, 1986). It has been found to have anti-inflammatory effects by decreasing the secretion of IFN-γ, TNF-α and IL-2 (Fidler et al., 1986). Mitoxantrone is effective for the treatment of RRMS and reduces the rate of clinical relapse and delays the progression of disability.

1.1.3.5.4 Natalizumab (Tysabri®)

This was the first monoclonal antibody developed for the treatment of RRMS. Natalizumab is a monoclonal antibody against the α4 chain of α4β1 integrin (very late antigen-4) (Engelhardt and Kappos, 2008) for which successful trials were performed (Tubridy et al., 1999; Miller et al., 2003). α4β1 is expressed on the surface of all leukocytes and the monoclonal antibody acts to prevent the binding of leukocytes to the ligand of α4β1, vascular cell adhesion molecule-1 (VCAM-1), at the blood brain barrier, thereby preventing the entry of lymphocytes into the CNS (Rice, Hartung and Calabresi, 2005; Johnson, 2007). Natalizumab was briefly withdrawn from the market after two patients in a trial receiving both Natalizumab

and IFN-β-1α developed progressive multi-focal encephalopathy (PML) another demyelinating disease (Kleinschmidt-DeMasters and Tyler, 2005; Langer-Gould et al., 2005) but has since been re-introduced. PML occurs as a result of reactivation of JC or BK virus which the majority of the population are infected with. Treatments that suppress the immune system have been have been found to increase the occurrence of PML (Matteucci et al., 2002; Jilek et al., 2010).

Other newer treatments for MS include Fingolimod (FTY 720; Gilenia®), Cladribine, Alemtuzumab (Campath-1H), Minocycline, Rituximab and Daclizumab (reviewed in (Vosoughi and Freedman, 2010). Fingolimod acts on sphingosine-1-phosphate (S1P) receptors and prevents the egress of the lymphocytes out of the secondary lymphoid organs (Massberg and von Andrian, 2006) thereby reducing the number of circulating lymphocytes and has proved to be beneficial in MS (Kappos et al., 2010). Cladribine (an analogue of purine nucleoside), Alemtuzumab (a humanised monoclonal antibody targeting CD52), Rituximab (an anti-CD20 antibody) and Daclizumab (a humanised monoclonal antibody against CD25) have all exhibited promise in trials for MS treatment (as reviewed in (Vosoughi and Freedman, 2010).

1.2 $CD4^+$ T cell subsets

During T cell activation, the fate of the $CD4^+$ T cells is determined by signal 3. The pro-inflammatory cytokine IL-1β has an important role in directly driving IFN-γ and IL-2 production by $CD4^+$ T cells, driving a pro-inflammatory response (O'Sullivan et al., 2006). Cytokine signalling drives the differentiation of naïve $CD4^+$ T cells towards their particular Th subset.

1.2.1 Cytokine signalling in $CD4^+$ T cells

There are numerous different cytokines able to elicit an inflammatory response and they are able to interact with each other to counter-regulate the immune response under different circumstances. First the downstream signalling pathways will be

described, followed by cytokine signalling by IL-12, IL-17 and IL-6, which are important in Th1 and Th17 signalling.

1.2.1.1 The downstream events in cytokine signalling

Receptor mediated cytokine signalling results in a series of down-stream activation events eventually leading to gene upregulation or downregulation. This process allows the extracellular cytokine signal to be transmitted to the nucleus for gene transcription. Cytokine signalling activates the intracellular janus kinase-signal transducer and activation of transcription (JAK-STAT) signalling pathway (see Figure 1.4). The JAKs are tyrosine kinases capable of phosphorylating the cytokine receptor after ligand binding to the surface of the receptor. This leads to the activation of specific STATs. The activation of STATs leads to the downstream expression of the Th lineage specific master regulator transcription factors T-bet (Th1), GATA3 (Th2), RORγt (Th17) and FoxP3 (Treg) (Zhu and Paul, 2010) (Hebenstreit, Horejs-Hoeck and Duschl, 2005). Importantly the suppressor of cytokine signalling (SOCS) family of proteins (SOCS1-7) which are produced after JAK-STAT signalling, act to inhibit the JAK-STAT signalling pathway (Krebs and Hilton, 2001) as summarised in Figure 1.4. The different SOCS proteins are able to bind either the cytokine receptor or the JAK itself to inhibit STAT signalling. In this way, JAK signalling induces transcription of both proinflammatory genes and SOCS proteins which subsequently act to inhibit the JAK-signalling, highlighting a level of negative feedback regulation of cytokine signalling.

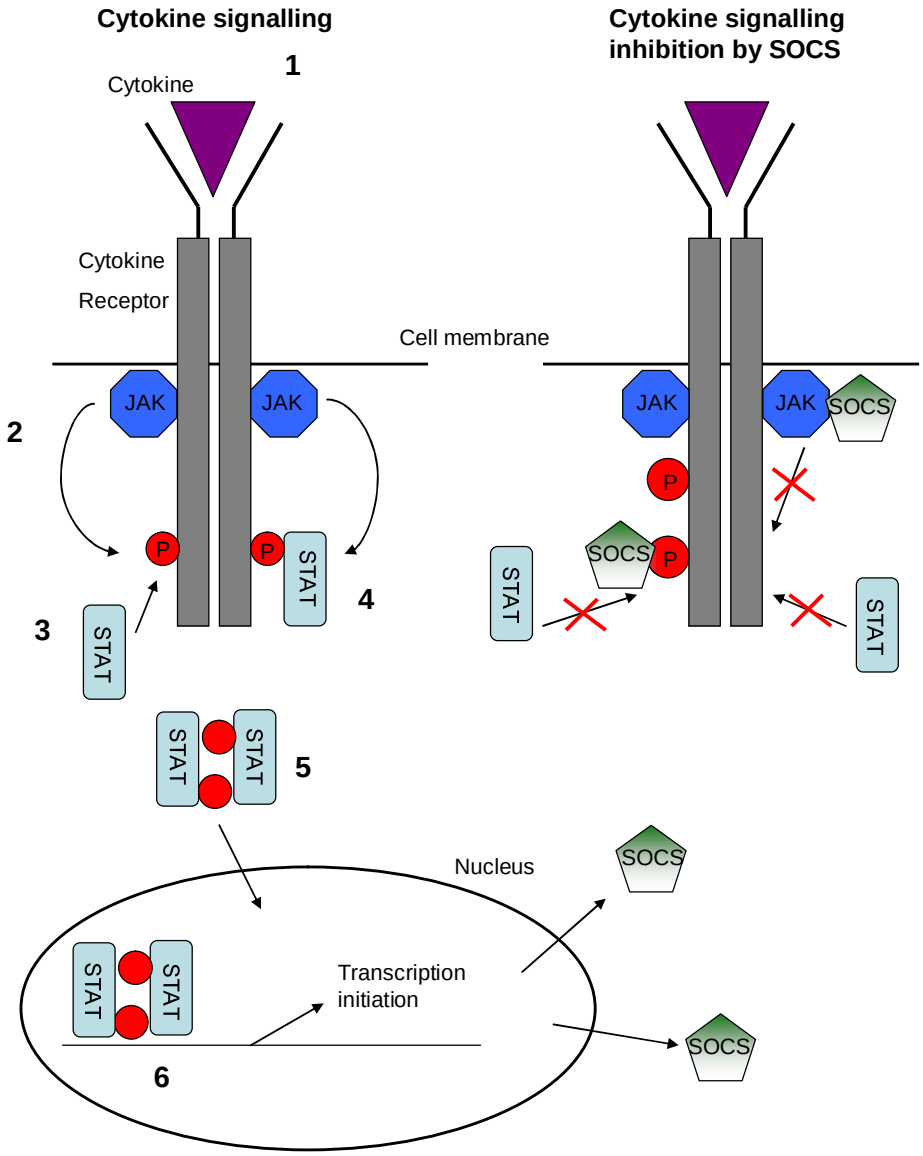

Figure 1.4 The JAK-STAT signalling pathway and its inhibition by the SOCS proteins.

Left shows cytokine signalling through the JAK-STAT pathway which results in gene transcription. Right shows the inhibition of the JAK-STAT pathway through SOCS proteins. 1) cytokine binds to the cytokine receptor; 2) binding of the cytokine activates JAK which then phosphorylates the intracellular domain of the cytokine receptor; 3) phosphorylation recruits the STAT proteins to the cytokine receptor; 4) JAK then phosphorylates the STAT protein; 5) the phosphorylation allows the STAT proteins to dimerise and migrate to the nucleus; 6) STAT dimers initiate the transcription of specific transcription factors and inflammatory cytokines as well as the production of SOCS. The SOCS proteins can then antagonise the JAK-STAT signalling pathway by binding to the phosphorylation sites on the cytokine receptor, preventing the binding of STATs, and by binding to JAKs directly preventing their phosphorylation of the cytokine receptor.

1.2.1.2 The IL-12 cytokine family

IL-12, which promotes Th1 differentiation (Hsieh et al., 1993), is a member of the IL-12 cytokine family which also includes IL-23, IL-27 and the recently identified cytokine, IL-35. All four cytokines are heterodimers consisting of an α chain (p19, p28 or p35) and a β chain (p40 or Ebi3). IL-12 is made up of subunits p40 and p35. The p40 chain can heterodimerise with p19 to form IL-23 (Oppmann et al., 2000). IL-27 is made up of α chain p28 and β chain Ebi3 (Pflanz et al., 2002) which was identified in Epstein-Barr virus infected B cells some years ago (Devergne et al., 1996). And finally, the newest member of the family, IL-35 is composed of the IL-12 α chain p35 and the β chain Ebi3 (Collison et al., 2007) as summarised in Figure 1.5. IL-12, IL-23 and IL-27 are all mainly produced by APCs after APC activation (Ma et al., 1996; Pflanz et al., 2002; Hibbert et al., 2003) whereas IL-35 has so far only been suggested to be produced in bioactive form by $FoxP3^+$ Tregs (Collison et al., 2007), although this remains controversial. The receptors for these cytokines are composed of various different receptor chains: IL-12Rβ1, IL-12Rβ2, IL-23R and gp130. The receptor for IL-12 is a pairing of IL-12Rβ1 and IL-12Rβ2 (Parham et al., 2002). The IL-23 receptor shares IL-12Rβ1 with IL-12 (Parham et al., 2002). IL-27 signals through gp130 and WSX-1 (also known as IL-27Rα) (Pflanz et al., 2004) and as yet, the receptor for IL-35 is unknown as shown in Figure 1.5. IL-12 signalling, as induced by IL-12 production by DCs and macrophages, results in promotion of IFN-γ and TNF-α production.

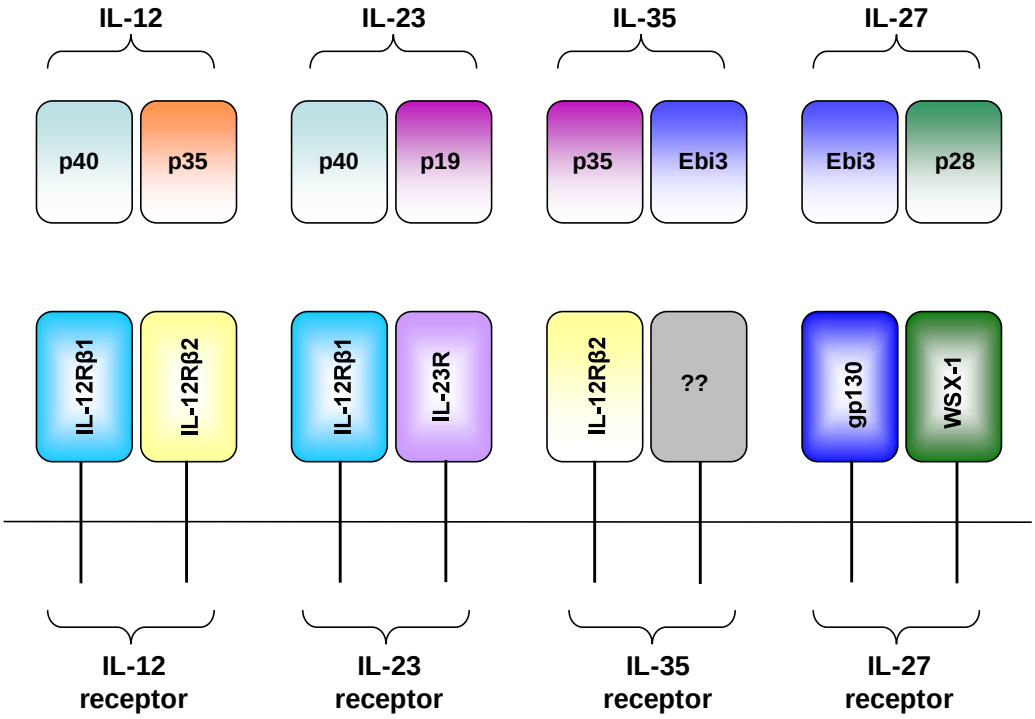

Figure 1.5 IL-12 cytokine family highlighting the sharing of subunits in the cytokines themselves, and the receptors.

The IL-12 family of cytokines is composed of IL-12, IL-23, IL-27 and IL-35. Each cytokine consists of an α chain and a β chain, and different combination pairings of the chains results in the formation of different cytokines. In the same way, IL-12, IL-23 and IL-35 share receptor subunits IL-12Rβ1 and IL-12Rβ2 although the second component of the IL-35 receptor is still unknown. In contrast, IL-27 has its unique receptor composed of gp130 and WSX-1. Adapted from Palmer and Weaver, 2010 (Palmer and Weaver, 2010)

1.2.1.3 IL-6 signalling

IL-6 belongs to the family of gp130 cytokines, of which IL-27 is also a member. All members have a four-helical protein structure and signal through a receptor complex which includes at least one subunit of the signal transducing receptor glycoprotein gp130 (Taga, 1997; Taga and Kishimoto, 1997; Pflanz et al., 2004).

IL-6 has been identified as being able to signal through two different pathways: classical signalling via membrane bound IL-6R on the surface of the target cell, or trans-signalling via a naturally occurring soluble IL-6R. In classical signalling, IL-6 binds to the membrane bound IL-6R; this binding leads to dimerisation of the IL-6R

and activation of gp130 (Taga and Kishimoto, 1997; Rose-John, 2001). Gp130 is expressed on all cells whereas IL-6R is not widely expressed, except on neutrophils and macrophages and some lymphocytes, therefore classical signalling is restricted to these cell types expressing IL-6R. During trans-signalling IL-6 binds to the naturally occurring soluble IL-6R. This IL-6/soluble IL-6R complex then activates gp130 leading to downstream signal transduction (Peters, Muller and Rose-John, 1998; Rose-John, 2001). In this way, cells that do not express IL-6R can still respond to IL-6 signalling through the action of the soluble IL-6R thereby increasing the spectrum of cell types on which IL-6 can have an effect.

1.2.1.4 The IL-17 cytokine family

IL-17 (or IL-17A) is part of the IL-17 family of cytokines. This is composed of several homologues of IL-17A, namely, IL-17B, IL-17C, IL-17D, IL-17E and IL-17 F (reviewed in Gaffen, 2009 (Gaffen, 2009). IL-17A and IL-17F are the most well characterised members of the family with apparent roles in inflammation and autoimmunity (Yang et al., 2008b; Awasthi and Kuchroo, 2009) although this is controversial (Haak et al., 2009) as will be discussed in later sections. The signals elicited by IL-17F are much weaker in terms of downstream gene activation compared to signalling through IL-17A. These two molecules can also dimerise to form IL-17A-IL-17F heterodimers which signal at an intermediate level (McAllister et al., 2005; Wright et al., 2007). The receptors of the IL-17 family, IL-17RA, IL-17RB, IL-17RC and IL-17RD also share ligands and can dimerise to form different receptors for each ligand. The exact nature of the receptor complex for each ligand is still not fully defined. The heterodimer between IL-17A-IL-17F has been found to signal through the IL-17RA/IL-17RC complex (Wright et al., 2008). IL-17E, also known as IL-25, is considered to be a Th2 promoting cytokine. It binds IL-17RB which pairs with IL-17RA (Claudio et al., 2009). It has been shown that mice deficient in both of these receptor subunits cannot respond to IL-17E (Rickel et al., 2008). The exact functions of IL-17B, IL-17C and IL-17D are still unclear. IL-17 signalling triggers the production of other pro-inflammatory cytokines, the release of chemokines which attract other cells, especially neutrophils, to sites of inflammation (reviewed in (Kolls and Linden, 2004)).

1.2.2 $CD4^+$ T cell differentiation

The main role of cytokine signalling is to trigger activation of downstream STAT proteins which each in turn play an important role in the differentiation of $CD4^+$ T cells towards a particular phenotype. During T cell activation, the naïve $CD4^+$ T cells can differentiate in to Th1, Th2 or Th17 cells. Originally, Mosmann described two distinct types of mouse $CD4^+$ T cell clones, Th1 and Th2 cells (Mosmann et al., 1986). These could be distinguished by their distinct cytokine production patterns as well as their differential expression of cell surface molecules. A new Th subset, distinct from Th1 and Th2 cells, has been recently defined, the Th17 lineage (Harrington et al., 2005; Park et al., 2005). Even more recently, two new subsets have been postulated, Th9 and Th22 cells (Dardalhon et al., 2008; Veldhoen et al., 2008b; Duhen et al., 2009; Trifari et al., 2009) although they remain to be confirmed as true Th subsets, as lineage specific transcription factors are yet to be identified. In contrast to these Th subsets, $CD4^+$ Treg cells play a crucial role in the regulation of an immune response, as both natural Treg cells (those developed in the thymus) and adapted Treg cells (aTreg; those differentiated from naïve $CD4^+$ T cells in the periphery). The role of each of these $CD4^+$ subsets will now be summarised. For a summary diagram of the main points see Figure 1.6 at the end of this section.

Each Th subset can be induced by TCR stimulation of naïve $CD4^+$ T cells *in vitro* in the presence of particular cytokine cocktails (with or without cytokine-neutralising antibodies). The different Th subsets also express distinct sets of chemokine receptors on their surface. This dictates their migration *in vivo*, and whether specific T cells can enter particular tissues before or during inflammation. This will be discussed in detail in section 1.4.

1.2.2.1 Th1 cells

Th1 cells make IFN-γ as their signature cytokine, as well as lymphotoxin (LT) and TNF-α. The production of these pro-inflammatory cytokines leads to the activation of macrophages at the site of inflammation. As such Th1 cells are important for host defence against intracellular pathogens, but they can also provoke unwanted

autoimmune inflammation. The activation of macrophages by IFN-γ is important for defence against intracellular microbes.

In vitro Th1 differentiation was found to be promoted by the addition of IL-12 and the neutralisation of IL-4 (Hsieh et al., 1993). The transcription factor T box expressed in T cells (T-bet) was discovered to be the key transcription factor of the Th1 lineage (Szabo et al., 2000). T-bet, a 530-amino acid protein with a 189-amino acid T box DNA-binding domain, is required for the stable activation of the IFN-γ gene and the induction of IFN-γ production. Importantly, ectopic expression of T-bet resulted in the induction of IFN-γ, and, if expressed in Th2-polarised cells, resulted in their switching of their phenotype towards Th1-IFN-γ production (Szabo et al., 2000). It has also been shown that although T-bet induces the production of IFN-γ, IFN-γ itself also promotes the induction of T-bet expression, highlighting an important autocrine loop in which IFN-γ is able to control the transcription factor that promotes its own production (Lighvani et al., 2001). IFN-γ is thought to activate STAT1, and this in turn induces the expression of T-bet (Afkarian et al., 2002). The mechanism by which T-bet upregulates IFN-γ is not fully elucidated. However, it is thought to be through the *Ifng* gene and through upregulating the IL-12Rβ2 expression, therefore increasing IFN-γ production through increasing the responsiveness to IL-12 (Mullen et al., 2001; Mullen et al., 2002). This correlates with the two step T-bet upregulation proposed recently (Schultz et al., 2009). Through mathematical modelling it was suggested that IFN-γ induced the initial expression of T-bet during T cell activation whereas the IL-12Rβ2 was suppressed at this stage. After TCR stimulation, T-bet upregulated IL-12Rβ2 and IL-12 was then able to feedback to maintain the T-bet expression (Schultz et al., 2009).

STAT4 also seems to be required for Th1 differentiation. STAT4-deficient mice display impaired Th1 responses together with an increase in Th2 responses, indicating STAT4 may act to suppress Th2 responses under normal conditions (Kaplan et al., 1996b; Thierfelder et al., 1996). Indeed, it has been found that GATA3, the key regulator of the Th2 lineage, suppresses Th1 responses directly through downregulation of STAT4, rather than acting upon T-bet, or the IL-12

receptor (Usui et al., 2003). STAT4 has a key role in Th1 differentiation as it can act directly to induce IFN-γ and induce the expression of IL-12Rβ2 as well as T-bet during the differentiation process (Usui et al., 2003; Usui et al., 2006).

IL-18 has also been implicated in differentiation towards a Th1 phenotype. It was first described as IFN-γ-inducing factor (IGIF) in T cells and NK cells (Okamura et al., 1995). IL-18 has been found to synergise with IL-12 for the induction of a Th1 IFN-γ-producing response (Micallef et al., 1996; Stoll et al., 1998) and is considered to augment the Th1 response (Robinson et al., 1997). It is thought to increase TNF-α (not IFN-γ), CCR4 and CCR7 (Ito et al., 2003).

Th1 cells were originally thought to be the key players in autoimmune disease. The pattern of cytokine production and chemokine expression on the autoreactive T cells found in MS patients had a Th1-phenotype (Merrill, 1992; Sorensen et al., 1999) and this was mimicked in murine EAE (Ando et al., 1989; Merrill et al., 1992), leading to the thought that MS and EAE were Th1-mediated. In addition, when MS patients were treated with IFN-γ this led to the exacerbation of disease (Panitch et al., 1987a; Panitch et al., 1987b), indicating IFN-γ can make MS worse. To further support this evidence, the loss of T-bet or STAT4 led to the profound abrogation of EAE (Chitnis et al., 2001; Bettelli et al., 2004). This evidence all pointed towards Th1 cells being the key pathogenic Th subset in induction of autoimmune inflammation. This will be discussed in more detail in a later section in light of more recent data.

In summary, the Th1 cells develop in the presence of IFN-γ, IL-12 and IL-18 and produce IFN-γ as their signature cytokine, as well as TNF-α and lymphotoxin. Th1 cells are T-bet$^+$, and their differentiation is tightly controlled by STAT1 and STAT4.

1.2.2.2 Th2 cells

Th2 cells make IL-4, IL-5 and IL-13 as their signature cytokines and do not produce IFN-γ or lymphotoxin. However, they can produce some TNF-α (Mosmann et al., 1991; Minty et al., 1993). Th2 cells have a very different role to Th1 cells, being required for the clearance of extracellular pathogens, for example helminth worm

infections, as well as having a detrimental role in allergy. This is due to the $CD4^+$ Th2 cell-dependent B cell antibody response after activation. The antibodies produced by the B cells after antigen presentation to the Th2 cells (IgG1 and IgE), and Th2 activation, provide the humoral response to eliminate extracellular pathogens and during allergic reactions (reviewed in (Maizels et al., 2004; Lindell et al., 2008). Th1-associated cytokines, IFN-γ and IL-12 inhibit the differentiation of naïve $CD4^+$ T cells into Th2 cells. To achieve successful Th2 polarisation, naïve $CD4^+$ T cells are activated in the presence of IL-4, leading to the phosphorylation of STAT6. This in turn leads to the induction of GATA3, the key transcription factor of Th2 cells (Zheng and Flavell, 1997), and the subsequent activation of Th2-cytokines IL-4, IL-5 and IL-13 (Zhang et al., 1997). GATA3 is also important for T cell development and naïve T cells express it at a low level. On activation and differentiation, Th2 cells upregulate GATA3 whereas cells differentiating toward the Th1 lineage downregulate its expression (Zhang et al., 1997).

STAT6 is important for Th2 differentiation and IL-4 signalling (Kaplan et al., 1996a; Takeda et al., 1996). The ectopic expression of STAT6 induces high levels of GATA3 expression as well as production of Th2-specific cytokines during the *in vitro* differentiation of Th1 cells (Kurata et al., 1999; Zhu et al., 2001) indicating its important role in the differentiation of Th2 cells, being able induce Th2 phenotype even in the presence of Th1-polarising conditions. STAT5, has also been found to be required for Th2 differentiation (Zhu et al., 2003). To support this, it was found that in the absence of either STAT5 isoform (STAT5a or STAT5b), Th2 differentiation is severely impaired, both *in vitro* and *in vivo* (Kagami et al., 2000; Kagami et al., 2001).

Th2 cells are not considered to be required in the induction of autoimmune disease. Myelin-responsive Th2 cells were not able to induce EAE after adoptive transfer, unless the Th2 cells were transferred into lymphopenic host mice (Baron et al., 1993; Lafaille et al., 1997; Jager et al., 2009).

In summary, Th2 cells produce IL-4, IL-5 and IL-13 as their signature cytokines. GATA3 is the master regulator of Th2 cells, which works together with STAT6 and STAT5 to ensure differentiation towards a Th2 phenotype and maintenance of the Th2 cytokine profile.

1.2.2.3 Th17 cells

In 2003, a new $CD4^+$ T cell subset, promoted by IL-23, was discovered (Aggarwal et al., 2003; Murphy et al., 2003). This new Th subset was later defined as the Th17 lineage (Langrish et al., 2005; Bettelli, Korn and Kuchroo, 2007) phenotypically distinct from the Th1 and Th2 lineages. In general, Th17 cells are required for clearance of pathogens and fungi at epithelial/mucosal barriers, and can also have a role to play in the induction of autoimmune inflammation (Littman and Rudensky, 2010).

Our understanding of the molecular requirements for Th17 differentiation is still being refined.

Th17 differentiation was shown to be inhibited by IFN-γ and IL-4, the key signature cytokines of Th1 and Th2 cells respectively. Th17 cells were shown to produce IL-17A and IL-17F, independently of STAT1, T-bet, STAT4 and STAT6 (reviewed in Harrington et al, 2006 (Harrington, Mangan and Weaver, 2006). The transcription factor retinoic acid-related orphan receptor gamma t (RORγt) was first discovered as an orphan nuclear receptor expressed on double-positive thymocytes (He et al., 1998) and found to be important for their survival during clonal selection. RORγt is also required for the development of lymphoid tissue inducer (LTi) cells and LTi-like cells which are required for development of lymphoid tissues (Sun et al., 2000; Eberl et al., 2004). Later RORγt was defined as the key transcription factor required for the differentiation of Th17 cells (Ivanov et al., 2006). RORγt-deficient mice lacked IL-17-producing T cells, highlighting that *in vivo* as well as *in vitro* differentiation of Th17 cells required the induction of RORγt expression (Ivanov et al., 2006). Another related nuclear receptor, RORα, has also been shown to be required for Th17 differentiation. The two transcription factors work together to promote Th17

differentiation more than when either is expressed alone (Yang et al., 2008d). To support this, it was shown that deficiencies in both RORα and RORγt severely impaired the IL-17 production or Th17 generation, and also resulted in the amelioration of disease in EAE (Yang et al., 2008d).

Initially it was thought that IL-23 was the key cytokine required for the differentiation of IL-17-producing pro-inflammatory T cells (Aggarwal et al., 2003; Langrish et al., 2005). However, it then emerged that IL-23 was not required for the differentiation of naïve T cells, but rather for the maintenance of the Th17 phenotype after their initial differentiation (Stritesky, Yeh and Kaplan, 2008). The IL-23R is not expressed on naïve $CD4^+$ T cells, but on differentiated Th17 cells, confirming that IL-23 can only act to maintain the Th17 phenotype once the cells are terminally differentiated. This IL-23 signalling is however essential for the development of a full Th17 response (McGeachy et al., 2009). Mice deficient in either IL-23 itself or the IL-23R exhibit an impaired ability to produce a significant Th17 response *in vivo* (Langrish et al., 2005; Awasthi et al., 2009; McGeachy et al., 2009). IL-23 also has the role of inducing the production of other effector cytokines by Th17 cells, for example, IL-22 (Liang et al., 2006). It has been determined that IL-6 and TGF-β were the key cytokines required for the differentiation of naïve $CD4^+$ T cells towards a Th17 phenotype (Bettelli et al., 2006; Mangan et al., 2006; Veldhoen et al., 2006; Korn et al., 2008).

IL-6 has been shown to be required for the *in vitro* and *in vivo* differentiation of Th17 cells (McGeachy et al., 2007; Zhou et al., 2007; Korn et al., 2008). In addition to this, IL-6-deficient mice are resistant to the induction of EAE highlighting a role for IL-6 signalling in autoimmune pathogenesis, possibly through the induction of a Th17 response, although roles in other pathways cannot be excluded (Okuda et al., 1998; Samoilova et al., 1998; Okuda et al., 1999).

The differentiation of human Th17 cells has proved to be slightly different to murine cells. In humans, IL-1β in combination with IL-6, TGF-β and IL-21 have been shown to be required for the differentiation towards a Th17 phenotype with the TGF-β

acting to inhibit Th1 cells (Manel, Unutmaz and Littman, 2008; Yang et al., 2008a; Santarlasci et al., 2009).

Lack of STAT3 resulted in the lack of IL-17-producing Th17 cells being generated, showing it is required for their differentiation, in both mouse and human cells (Harris et al., 2007; Yang et al., 2007; Milner et al., 2008). STAT3 functions by binding to the *Il17* gene and also to the *Il21* gene, (Chen et al., 2006b; Wei et al., 2007) and induces the expression of both RORγt and IL-23R (Nurieva et al., 2007; Zhou et al., 2007). IL-6 has the role of activating STAT3 and this is turn also acts to downregulate FoxP3 expression, thereby suppressing the induction of $FoxP3^+$ Treg, and highlighting the important role IL-6 has in the balance between Th17 and Treg differentiation (Yang et al., 2007). STAT5, a Th2-associated STAT protein, has an inhibitory effect on Th17 cell differentiation (Laurence et al., 2007) which highlights why IL-2 (which triggers STAT5 activation) can have a negative effect on the Th17 cell generation. However, once Th17 cells are terminally differentiated, it seems that IL-2 signalling through STAT5 is required for their maintenance and expansion (Amadi-Obi et al., 2007).

On discovery of IL-23 and the Th17 lineage there was a paradigm switch towards Th17 cells being the key inducers of autoimmune disease. This will be discussed in full in a section 1.3.5.2.

1.2.2.4 $CD4^+$ T regulatory cells

A subpopulation of T cells has long been known to be a vital player in regulation and maintenance of peripheral tolerance. Initially it was found that in mice the depletion of the $CD5^+$ population resulted in multi-organ inflammation (Sakaguchi et al., 1985). The phenotype of these 'regulatory cells' was gradually refined over time and defined as $CD4^+$ $CD25^+$ Treg (Sakaguchi et al., 1995). Similar experiments were performed in the rat (as reviewed in (Powrie et al., 1991). The transfer of CD25-depleted $CD4^+$ cells into lymphopenic mice resulted in the induction of autoimmune disease of several forms, i.e. thyroiditis, insulitis and polyarthritis (Sakaguchi et al., 1995) due to lack of suppression of the inflammatory response. Reciprocally, co-

transfer of $CD4^+$ $CD25^+$ T cells provided protection against autoimmune disease, highlighting the regulatory potential of $CD4^+$ $CD25^+$ T cells (Sakaguchi et al., 1995) in maintaining self tolerance. Treg develop in the thymus. This was determined after it was observed that neonatal thymectomy 3 days after birth resulted in a block in the presence of $CD4^+$ $CD25^+$ cells in the periphery (Sakaguchi et al., 1996).

Later, Foxp3 was identified to be the key transcription factor required by Treg for their development and suppressive function (Fontenot, Gavin and Rudensky, 2003; Hori, Nomura and Sakaguchi, 2003). To support this, scurfy mice deficient in functional FoxP3, due to a mutation in the gene, have severe lymphoproliferative disease (Brunkow et al., 2001). Similarly, the human autoimmune syndrome immunodysregulation polyendocrinopathy enteropathy X-linked syndrome (IPEX) is caused by mutations in the human homolog of the *Foxp3* gene (Bennett et al., 2001), highlighting its importance in the control of immune homeostasis.

It was later determined that FoxP3 expression can also be induced in naïve $FoxP3^-$ $CD4^+$ T cells either *in vivo* (as defined in 1.2.2) or *in vitro* (induced Treg; iTreg) in the presence of TGF-β (Chen et al., 2003; Fu et al., 2004). As yet, it is not possible to distinguish between nTreg and aTreg, as they both express FoxP3. The methylation status of the *foxp3* locus, however, has found to be a marker of FoxP3 expression stability in Treg. nTreg, which remain stable and continue to express FoxP3, display complete demethylation, whereas TGF-β-induced Treg exhibit only partial demethylation, despite high expression of FoxP3 (Floess et al., 2007). These iTreg display plasticity in their phenotype and can lose FoxP3 expression as well as suppressive function after re-stimulation in the absence of TGF-β (Floess et al., 2007).

Treg have been shown to elicit their regulatory suppression on multiple 'layers,' including: 1) within the lymph node during the steady state, by DC conditioning leading to weak stimulation and no effector response during an inflammatory response; 2) in the lymph node during an inflammatory response and exposure to self antigen, Treg proliferate and are therefore able to counter the development of a T

effector response; 3) some of these Treg leave the lymph node and migrate to the target organ and initiate the resolution of the inflammation by suppression of the inflammatory response (reviewed in (O'Connor and Anderton, 2008; Vignali, Collison and Workman, 2008) .

Interestingly, it has been shown that to control Th1-mediated inflammation Treg adopt the expression of T-bet, in response to IFN-γ (Koch et al., 2009). T-bet$^+$ FoxP3$^+$ Treg were better able to control the expansion of CD4$^+$ T-bet$^+$ Th1 effector cells than T-bet$^-$ FoxP3$^+$ Treg were. In the same way, T-bet$^-$ FoxP3$^+$ Treg were better able to control Th2 or Th17 mediated inflammation compared to T-bet$^+$ Treg. This suggested that in order to suppress a particular T helper response, Treg may partially adopt the phenotype of the response they are attempting to control.

The expression of *IFN regulatory factor 4 (Irf4)* in Treg has been shown to be important for their suppression of a Th2 response (Zheng et al., 2009). Similarly, STAT3 expression in Treg has been found to be the key factor required to facilitate Treg suppression of a Th17 response (Chaudhry et al., 2009). Therefore, Treg appear able to suppress Th1, Th2 and Th17-mediated responses through the expression of key transcription factors associated with each T effector subset and this highlights how Treg are able to effectively control different immune responses.

The potential of Treg therapy in autoimmune disease is a subject of intense investigation. Either the expansion of Treg *in vivo* or the transfer of *in vitro* expanded nTreg or iTreg could provide a means to control autoreactive T cells *in vivo*. However, the plasticity of Treg has been brought into question recently, with reports that they can begin to produce proinflammatory cytokines i.e. IL-17 (Lochner et al., 2008; Yang et al., 2008c).

1.2.2.5 The relationship between Th17 cells and Treg

Th17 cells and Treg share a close developmental relationship. Pro-inflammatory Th17 cells require anti-inflammatory TGF-β for their differentiation, as do aTreg in the periphery. The presence of TGF-β alone leads to the development of FoxP3$^+$

aTreg following TCR stimulation. In contrast, if TGF-β is found together with pro-inflammatory IL-6, Th17 cells are generated, thereby highlighting the importance of IL-6 in suppressing the expression of FoxP3 and promoting the development of Th17 cells at the same time (Bettelli et al., 2006). It is interesting how TGF-β is able to differentiate two opposing phenotypes of T cells. It is thought that low concentrations of TGF-β favour Th17 differentiation in the presence of IL-6, whereas high concentrations of TGF-β favour differentiation towards aTreg phenotype, suppressing the expression of IL-23R (Zhou et al., 2008). Functionally, Treg and Th17 cells also exhibit a reciprocal relationship. RORγt and RORα and FoxP3 are able to bind to each other and inhibit the others' activity (Zhou et al., 2008). Therefore, RORγt and RORα act to suppress FoxP3 and, reciprocally, FoxP3 acts to suppress RORγt and RORα. However, the presence of other Th17-associated cytokines, for example IL-6, IL-21 and IL-23 had the effect of removing the FoxP3-mediated inhibition of RORγt and allowing the promotion of differentiation towards a Th17 phenotype (Zhou et al., 2008). This highlights the importance of the surrounding cytokine environment in determining the outcome of Treg or Th17 balance. Importantly, retinoic acid (all-trans retinoic acid, ATRA) inhibits Th17 differentiation and, instead, promotes FoxP3 expression and Treg differentiation (Elias et al., 2008).

1.2.2.6 Th9 cells

Another possible Th subset, Th9, has recently been proposed. These are induced in the presence of IL-4 and TGF-β. The IL-4 suppresses TGF-β-driven expression of Foxp3 and results in a $FoxP3^-$ T effector cell that produces IL-9 and IL-10 (Dardalhon et al., 2008; Veldhoen et al., 2008b). These Th9 cells do not express other lineage-specific transcription factors, for example, T-bet, GATA3, RORγt or FoxP3. However, a Th9 lineage-specific transcription factor is yet to be identified and Th9 cells are therefore still to be confirmed as a true Th subset.

IL-9 and IL-10 have previously been associated with the Th2 lineage. However, Th9 cells produce very low levels of other Th2 cytokines, and produce high amounts of IL-9 which has a pro-inflammatory role (Li and Rostami, 2010). Th9 cells have been

shown to have a T effector phenotype, rather than a regulatory phenotype, despite their production of IL-10 (Jager et al., 2009). Th9 cells were able to induce disease, both in the EAE model (Jager et al., 2009) and in a model of colitis (Dardalhon et al., 2008).

1.2.2.7 Th22 cells

Production of IL-22 was described in differentiated Th17 cells (Kreymborg et al., 2007). However, Th22 cells have now been suggested to be a distinct subset able to produce IL-22, but neither IL-17 nor IFN-γ (Duhen et al., 2009; Trifari et al., 2009). Their differentiation is promoted in the presence of IL-6 and TNF-α (Duhen et al., 2009; Trifari et al., 2009). Human Th22 cells have been shown to express skin-homing receptors CCR4 and CCR10 which implicate them skin homeostasis and inflammatory pathology (Duhen et al., 2009; Eyerich et al., 2009).

1.2.2.8 Tr1 cells

Tr1 cells are another $CD4^+$ regulatory T cell subset, producing IL-10 and IFN-γ, however Tr1 cells are $FoxP3^-$ compared to the $FoxP3^+$ Tregs (Vieira et al., 2004). Tr1 cells are induced in the presence of IL-10 and IL-27 (Groux et al., 1997; Awasthi et al., 2007; Fitzgerald et al., 2007) and may have an important role to play in regulating autoimmune disease, especially with respect to regulating Th17 responses (Batten et al., 2006).

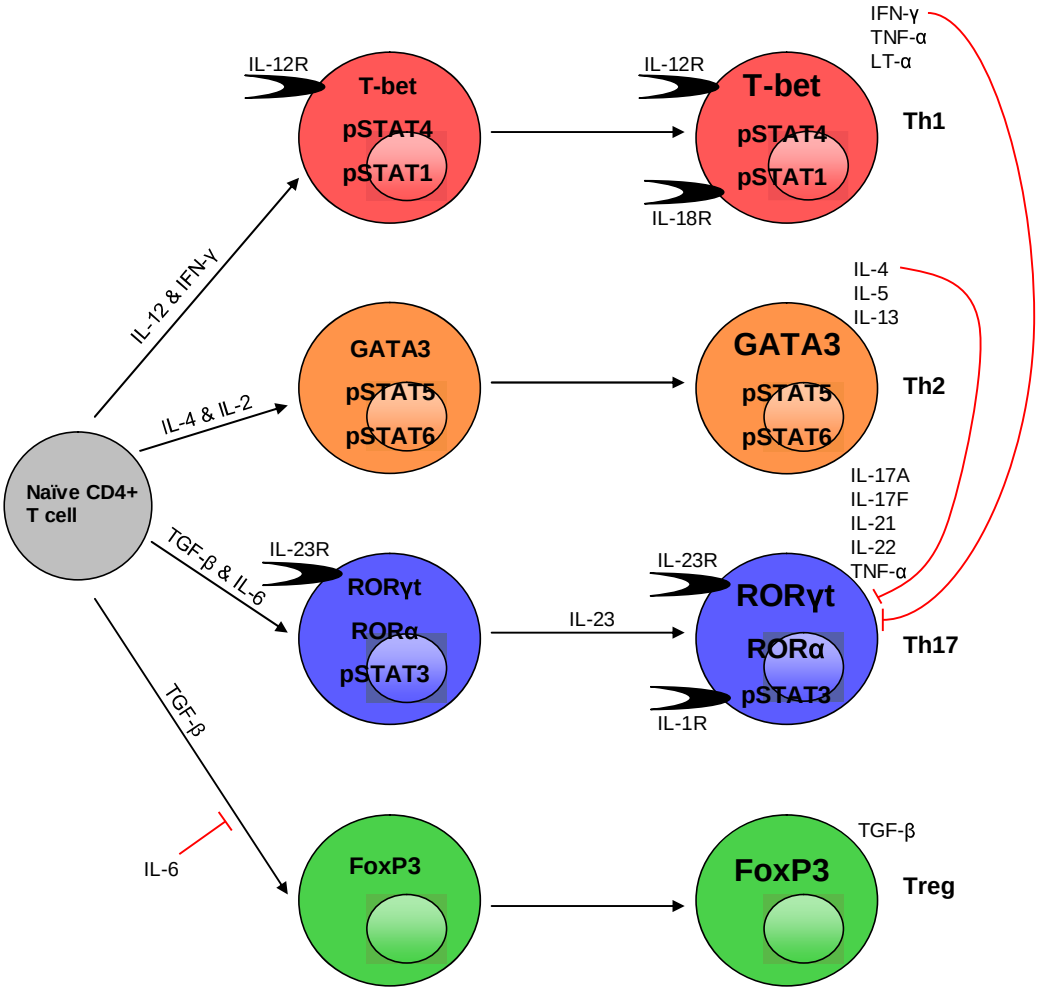

Figure 1.6 Differentiation of the key CD4$^+$ T cell subsets, and their relationship with each other.

Th differentiation from naïve CD4$^+$ T cells in the presence of polarising cytokines. Highlights the cytokines required for differentiation and maintenance of Th1, Th2 and Th17 lineages, and the aTreg and iTreg, and the relationship and interplay between the different subsets.

1.3 EAE: The primordial CD4$^+$ T cell-driven autoimmune disease model

1.3.1 History

The origin of EAE dates back to when spinal cord inflammation was induced in rabbits by injection with human spinal cord homogenate (Koritschoner and Schweinburg 1925). Since then, this has been reproduced in primates (Rivers et al 1933) and rodents, and EAE has now developed into a robust model of MS. EAE recapitulates most of the main features of inflammation observed in MS, and the study of EAE has been extremely useful in terms of understanding basic T cell biology, tolerance, inflammation and autoimmune disease. Importantly, in terms of what EAE has taught us about MS, two current therapies for MS were originally developed after observations that they were efficacious in EAE: glatiramer acetate and natalizumab (anti-α4β1-integrin) (Yednock et al., 1992; Ben-Nun et al., 1996; Teitelbaum et al., 1996). Other current treatments of MS, IFN-β and Mitoxantrone, were initially developed independently of EAE. Due to the heterogeneity of both MS and EAE it is unfortunately not possible to predict whether a therapeutic showing promise in EAE will be as successful in treating disease when translated to MS.

1.3.1.1 EAE as a CD4$^+$ T cell-driven disease

T cells were implicated in EAE when it was observed that disease could be initiated by the transfer of lymph node cells from donor rats sensitised to spinal cord, into non-immunised recipient rats (Paterson, 1960), indicating that the disease was cell-mediated and not antibody mediated. Neonatal thymectomy resulted in the prevention of EAE development (Arnason et al., 1962) implicating the importance of T cells in disease induction. Subsequently, CD4$^+$ T cells specifically were implicated in disease pathogenesis after the observation that EAE was inhibited by the depletion of CD4$^+$ T cells prior to transfer to recipient mice, whereas the depletion of CD8$^+$ T cells had no effect on disease induction (Pettinelli and McFarlin, 1981). The transfer of disease with CNS autoantigen specific CD4$^+$ T cells gave the ultimate evidence for the importance of CD4$^+$ T cells in mediating disease in rats (Ben-Nun, Wekerle and Cohen, 1981). Similarly this was also determined in mice (Zamvil et al., 1985).

1.3.1.2 CNS autoantigens of EAE and TCR transgenic models

Since the initial induction of EAE with the use of brain and spinal cord homogenate, several CNS autoantigens have been defined and the main ones are outlined in Table 1.1, along with the corresponding mouse strain with which they can be used. The autoantigens myelin basic protein (MBP) (Zamvil et al., 1985; Zamvil et al., 1986), proteolipid protein (PLP) (Tuohy et al., 1989; Greer et al., 1992) and myelin oligodendrocyte glycoprotein (MOG) (Mendel, Kerlero de Rosbo and Ben-Nun, 1995) have been shown to induce EAE upon immunisation in susceptible mice strains.

Autoantigen	Peptide	Strain	TCR Transgenic
Myelin oligodendrocyte glycoprotein (MOG)	MOG_{35-55}	C57BL/6 (H-2^b)	2D2 (Bettelli et al., 2003)
Myelin basic protein (MBP)	MBP (Ac1-9) MBP(Ac1-11) MBP(Ac1-17)	B10.PL (H-2^u), PL/J (H-2^u)	Tg4 (Liu et al., 1995) αβ-transgenic (Goverman et al., 1993) MBP-TCR Tg^+ (Hardardottir, Baron and Janeway, 1995)
Proteolipid protein (PLP)	$PLP_{139-151}$	SJL (H-2^s)	5B6 (Waldner et al., 2000)

Table 1.1 CNS autoantigens of EAE

The autoantigen, relevant peptide and corresponding mouse strain and TCR transgenic in key EAE models. This table highlights MOG, MBP and PLP autoantigens. MOG and MBP are the antigens used within this thesis.

Early EAE experiments were performed on susceptible strains of mice using MBP immunisation, or transfer of MBP-reactive cells into naive recipients e.g. B10.PL or PL/J mice. C57BL/6 mice were found to be largely resistant to the induction of EAE. However, after the discovery of the autoantigen MOG, it was found that MOG could induce the development of chronic EAE in C57BL/6 mice on immunisation (Mendel, Kerlero de Rosbo and Ben-Nun, 1995) using either recombinant MOG, or a peptide of MOG ($pMOG_{35-55}$). This development was a big step forward as it then allowed

for the assessment of EAE in the many gene knock-out mice on the C57BL/6 background. MOG is also implicated in the pathogenesis of MS with human T and B cells being responsive to MOG (Sun et al., 1991; Kerlero de Rosbo et al., 1993). There are different models of EAE: monophasic EAE, chronic relapsing remitting EAE and spontaneous EAE, each recapitulating different aspects of MS. The autoantigen used to induce disease has a part in determining the disease phenotype and pattern of lesion distribution in the CNS (Berger et al., 1997). Also the development of the TCR transgenic mice, for example the MOG-reactive 2D2 mice (Bettelli et al., 2003) and the MBP-reactive Tg4 mice (Liu et al., 1995) has been a great advantage as it allows the investigation of the development of naïve TCR-transgenic T cells into inflammatory T effector cells both *in vitro* and *in vivo*. The Tg4 mice express a TCR which recognises the immuno-dominant Ac1-9 peptide of MBP (4Lys) when presented in the context of I-A^u. The Tg4 mice have been crossed with CD45.1 mice to produce Tg4 CD45.1 mice, which allow Tg4 cells to be tracked *in vivo* when transferred.

EAE can be induced as both 'active' EAE and 'passive transfer' EAE. Active EAE is the immunisation of host mice with the autoantigen as defined above, mixed together with complete Freund's adjuvant (CFA). Naïve $CD4^+$ T cells are activated *in vivo* and migrate towards the CNS to elicit an inflammatory response. In contrast passive EAE is the transfer of pre-activated autoantigen reactive encephalitogenic T cells into susceptible host mice. The transferred cells can be either *in vivo* primed non-transgenic donor cells, or *in vitro* primed TCR transgenic donor cells. Within the chronic form of EAE extensive demyelination in the CNS is evident. During passive transfer EAE however, the demyelination is not extensive. Instead, the activated encephalitogenic T cell interacts with the MHC-II/peptide complex presented by the resident APCs in the CNS (for example microglia or astrocytes), and this results in the development of an inflammatory response within the micro-environment. This is mediated by the production of pro-inflammatory cytokines, increased permeability of the blood brain barrier as well as the recruitment of other inflammatory T cells or monocytes to the CNS. This inflammatory pathology is associated with the observed development of disease.

In this thesis the passive transfer model of EAE is mainly used, using both pMOG-reactive *in vivo* primed cells and the *in vitro* primed MBP-reactive TCR transgenic Tg4 cells. These donor cells, as mentioned above, are traceable *in vivo*, due to the presence of congenic markers (CD45 or CD90). Passive transfer models have the advantages of 1) allowing the generation of both *in vivo* primed and *in vitro* primed encephalitogenic T cells; 2) determining the encephalitogenic potential of different Th subsets *in vivo*; 3) being able to track the donor T cells to determine their migration potential as well as their phenotypic stability *in vivo*.

The use of the TCR transgenic model allows for the investigation of the role of different T cell subsets in a system in which all the T cells concerned are specific for a particular antigen and is therefore a 'cleaner' system. The polyclonal model is also important to use as it investigates the same question in a system in which all the T cells are not specific for a particular antigen which would be more realistic as compared to the TCR transgenic model. The use of C57BL/6 mice for the polyclonal model (as opposed to B10.PL) allowed for the use of gene-knock-out host mice which were on the C57BL/6 background, and is also the general approach used in the Anderton Lab as well as in other EAE research groups.

1.3.1.3 B cells in EAE

B cells have the ability act in various different ways in EAE. They can produce pathogenic autoantibodies; B cells can act as APC and enhance the activation of the autoreactive T cells; or they can also act in a regulatory fashion. This has been demonstrated by autoantibodies which can increase the severity of disease (Litzenburger, 1998). In contrast, IL-10 production by B cells has been shown to be regulatory in EAE (Fillatreau et al., 2002). B cells have also been shown to have a regulatory role, controlling self-reactive T cells, by other mechanisms. Firstly, they have recently been shown to have a role to play in the induction of negative selection of self-reactive T cells in the thymus (Frommer and Waisman, 2010). Secondly, antigen presentation by B cells has been shown to result in induction of tolerance to EAE by the upregulation of coinhibitory molecules including CTLA-1 and PD-1 (Frommer et al., 2008).

1.3.2 Nature of pathogenic CD4$^+$ T cells in EAE

1.3.2.1 EAE and MS as Th1 mediated diseases

EAE was originally considered to be a Th1-mediated autoimmune disease due to the Th1-phenotype of the encephalitogenic T cells (Ando et al., 1989). This correlated with MS in that the inflammatory T cells detected in the peripheral blood and CSF of MS patients had a predominantly Th1 phenotype (Balashov et al., 1999; Strunk et al., 2000; Teleshova et al., 2002). Treatment of MS patients with IFN-γ led to the exacerbation of disease (Panitch et al., 1987a; Panitch et al., 1987b), suggesting that IFN-γ was able to worsen disease. In addition, inhibition of IL-12-driven Th1 differentiation and IL-12 signalling led to the abrogation of EAE (Bright et al., 1998a). The expression of IL-12p40 mRNA was observed to be upregulated specifically in the brain and spinal cord of EAE mice and came down to baseline levels on resolution of disease (Bright et al., 1998b). The treatment of mice with an anti-IL-12 antibody (Bright et al., 1998b) led to the inhibition of disease, once again pointing towards the importance of IL-12 and a Th1 response in the induction of EAE.

However, there were always those conflicting data that IFN-γ-deficient mice as well as IFN-γR-deficient mice were still susceptible to EAE, and actually exhibited more severe disease than their wild-type counterparts (Ferber et al., 1996; Willenborg et al., 1996). In the same way, mice treated with antibodies against IFN-γ also exhibited severe disease (Billiau et al., 1988; Lublin et al., 1993). So, the apparent role of a Th1 response did not correlate with the exacerbated disease observed in the absence of IFN-γ, the key Th1 cytokine.

1.3.2.2 The move from Th1 to Th17 mediated EAE and MS

IL-23 is a heterodimeric cytokine composed of the IL-23 unique subunit p19, and the p40 subunit which is shared with IL-12 (Oppmann et al., 2000). The discovery of IL-23 and its structure highlighted that previous studies on IL-12 using the anti-IL-12p40 antibody, had in fact been blocking both IL-12 and IL-23. From this initial discovery of IL-23, research then focused on trying to determine the role of IL-23 in

autoimmune pathogenesis. IL-23 was found to be more important than IL-12 in the pathogenesis of EAE (Cua et al., 2003). This was determined by generating specific IL-12-deficient ($p35^{-/-}$) and IL-23 deficient ($p19^{-/-}$) mice for the comparison with the previously used double-deficient ($p40^{-/-}$) mice. IL-12 deficient mice were still susceptible to MOG-induced EAE, whereas disease was abrogated in IL-23 deficient mice (either IL-23 alone, or double-deficient mice) (Cua et al., 2003). This suggested that IL-23 had a more important role to play in autoimmune disease induction in the CNS. The same result was observed in collagen induced arthritis (CIA), a murine model of human rheumatoid arthritis (RA) (Murphy et al., 2003) suggesting IL-23 was important for autoimmune disease induction on a wider scale.

It was then reported that IL-23 could drive an IL-17-producing T cell population and that those IL-17-producing cells appeared to have a pro-inflammatory phenotype, inducing EAE (Aggarwal et al., 2003; Langrish et al., 2005). IL-23 appeared to promote a cell population that produced IL-17A, IL-17F, IL-6 and TNF-α and, on adoptive transfer, these cells were able to target the CNS and induce EAE (Langrish et al., 2005). This observation led to the description of Th17 cells as a novel Th subset as described in section 1.2.2.3. To add weight to the importance of IL-23 in autoimmune pathogenesis, treatment of mice with an anti-IL-23 antibody led to the amelioration of EAE (Chen et al., 2006a).

To investigate the role of IL-17 and the newly discovered Th17 lineage EAE was induced in IL-17A-deficient mice. Disease was found to be reduced, but not completely abrogated (Komiyama et al., 2006). IL-17 was then hailed as the key proinflammatory cytokine in the pathogenesis of EAE. However, as disease was not completely ameliorated in its absence, this was a large assumption.

As mentioned in section 1.2.2.3, IL-23 was later shown to be dispensable for the differentiation of naïve $CD4^+$ T cells towards a Th17 phenotype, but was required for the maintenance and survival of Th17 cells (Stritesky, Yeh and Kaplan, 2008). Importantly, polarisation of ‘Th17’ cells using IL-23 alone led to the generation of a mixed population of IFN-γ and IL-17-producers (O'Connor et al., 2008). Therefore,

in previous publications that used IL-23-driven 'Th17' populations to induce disease it was unclear as to which population, IFN-γ^+ or IL-17^+, had actually induced inflammation (Harrington et al., 2005; Park et al., 2005). IL-6 and TGF-β were identified to be the key cytokines required to generate Th17 cells, and IL-23 required to maintain and stabilise their IL-17-producing phenotype (Veldhoen et al., 2006; McGeachy et al., 2007). In the absence of IL-1β signalling, another cytokine shown to be important for Th17 differentiation, EAE was significantly reduced in severity due to fewer Th17 cells after immunisation of IL-1R-deficient mice (Sutton et al., 2006).

Despite the evidence pointing towards Th17 cells being a key pathogenic subset in autoimmune disease, recent data have shown that IL-17 itself is not required for the induction of EAE. The over-expression of IL-17A, specifically on T cells, or its complete loss, has no effect on EAE induction (Haak et al., 2009). In the same way, the loss of IL-17F, which may compensate for IL-17A, had no effect on EAE induction, and antibody blockade of IL-17A in IL-17FKO mice did not induce resistance to EAE.

In MS, IL-17-producing cells have been shown to promote the disruption of the BBB through interactions with IL-17R which is expressed on the endothelial cells making up the BBB in MS lesions (Kebir et al., 2007). In addition, pro-inflammatory IL-6, one of the main promoters of Th17 differentiation, is upregulated in active lesions in MS patients, as compared to inactive lesions (Baranzini et al., 2000; Mycko et al., 2004) suggesting possible upregulation of a Th17 response in these areas. A clinical trial using a monoclonal antibody against the p40 subunit of IL-12 and IL-23 (thereby neutralising both IL-12 and IL-23 signalling) named Ustekinumab was used to treat patients with relapsing-remitting MS (Segal et al., 2008). This however had no beneficial effects on MS patients as compared to the placebo controls. The effect of neutralising IL-17 specifically in MS is yet to be determined.

1.3.2.3 T cell transcription factors in EAE

T-bet, the master transcription factor of Th1 cells has an important role in EAE induction. T-bet-deficient mice have consistently been reported to be resistant to EAE (Bettelli et al., 2004; Nath et al., 2006). Silencing T-bet signalling through the use of small interfering RNAs (siRNA) had an adverse effect on Th1-mediated processes (Lovett-Racke et al., 2004). Silencing T-bet reduced IFN-γ production as well as STAT1 expression levels. Importantly, transfer of T-bet siRNA-transfected myelin responsive T cells led to the reduction in EAE incidence and severity (Lovett-Racke et al., 2004).

Extending this, T-bet expression was recently found to be required within encephalitogenic T cells in a passive transfer model (Yang et al., 2009). IFN-γ-producing cells could be divided into those that were T-bet$^+$ RORγt$^-$ and a smaller population that were T-bet$^+$ RORγt$^+$ (Abromson-Leeman, Bronson and Dorf, 2009). The T-bet$^+$ RORγt$^-$ cells produced IFN-γ and no IL-17. However, the T-bet$^+$ RORγt$^+$ cells were able to produce both IFN-γ and IL-17, highlighting the plasticity of these cells. The double positive cells were able to switch between IFN-γ and IL-17 production. Interestingly both populations, irrespective of RORγt expression, were able to induce EAE to the same extent indicating a role for T-bet, but not RORγt, in EAE induction (Abromson-Leeman, Bronson and Dorf, 2009). In contrast, the induction of EAE in mice with RORγt-deficient T cells resulted in reduced severity of disease (Ivanov et al., 2006).

Another recent publication has highlighted how the expression of T-bet is required for encephalitogenicity, rather than the end products of Th1 or Th17 pathways, i.e. IFN-γ or IL-17. Encephalitogenic cells from spontaneous EAE and actively induced EAE were investigated for the expression of various markers (Yang et al., 2009). T-bet was found to be consistently expressed on the encephalitogenic T cells, whether they were producing IFN-γ or IL-17, highlighting that the T-bet expression was the key factor for disease pathogenesis (Yang et al., 2009).

STAT1, another key transcription factor required for the generation of the Th1 lineage, has been shown to be dispensable for the induction of EAE as STAT1-deficient mice are fully susceptible to EAE induction (Bettelli et al., 2004) whereas STAT4 deficient mice are protected (Thierfelder et al., 1996).

1.3.2.4 T regulatory cells in EAE

It has long been known that Treg have a role in the inhibition of inflammation in EAE (Kohm et al., 2002; Zhang et al., 2004). Transferred polyclonal Treg have been shown to inhibit the development of disease by affecting the priming of the autoreactive T cells within the lymph node (Kohm et al., 2002). However, the accumulation of IL-10-producing $FoxP3^+$ Treg within the CNS itself has also been observed during EAE (McGeachy, Stephens and Anderton, 2005). This accumulation correlated with disease resolution and the Treg, when recovered from the CNS were able to suppress T effector cells *in vitro* as well as transfer protection to recipient mice.

Interestingly, the increase in the number of $FoxP3^+$ Treg within the CNS during recovery from EAE is due to their activation and proliferation within the target organ (O'Connor, Malpass and Anderton, 2007) as opposed to increased infiltration of Ag-reactive Treg into the CNS previously primed in the periphery. Importantly, these CNS-derived Treg were able to suppress an IFN-γ response, but not an IL-17 response, from the CNS derived effector cells (O'Connor, Malpass and Anderton, 2007). This result led on to the central hypothesis for this thesis project, which will be explained in section 1.5.

The frequency of $CD4^+$ $CD25^{hi}$ Treg in MS patients in the peripheral blood was found to be similar to that in healthy individuals (Viglietta et al., 2004) indicating that there was no lack of Treg in MS patients. Interestingly, the function of these Treg in MS patients was found to be impaired. $CD4^+$ $CD25^{hi}$ Treg isolated from healthy controls were able to suppress the proliferation of $CD4^+$ $CD25^-$ responder T cells; however the Treg isolated from MS patients were unable to suppress proliferation (Viglietta et al., 2004). Therefore, Treg isolated from the blood of MS

patients, appear to be present in comparable levels to those in the healthy controls however they lack efficient regulatory function. This could account for the lack of control of the autoreactive T cells in the periphery, resulting in disease onset.

To investigate the potential role of Treg as a cellular therapy for EAE, and eventually MS, it is important to firstly determine whether adoptively transferred Treg are able to have a suppressive effect on on-going disease rather than only when transferred before the induction of disease. It is also important to determine whether antigen-specificity of the Treg is important. It may be essential to use antigen-specific Treg in patients to ensure an effect on auto-aggressive T cells specifically and to prevent a global immunosuppressive effect. Promising results have been found. Myelin-specific $CD4^+$ $CD25^+$ Treg from TCR transgenic mice were able to suppress disease on transfer into mice with chronic EAE (Stephens, Malpass and Anderton, 2009). Interestingly this was not observed with polyclonal Treg indicating that the antigen-specificity is important.

1.3.3 Classical versus atypical EAE

Classically, EAE is characterised by inflammation of the spinal cord in particular, with no overt involvement of the brain-stem or cerebellum (Cross, O'Mara and Raine, 1993; Archambault et al., 2006). In contrast, a different form of EAE has been described, termed atypical, or non-classical, EAE. This is characterised by inflammation within the cerebellum and brain-stem, and not the spinal cord and is particularly evident when the pathogenic T cells lack IFN-γ (Wensky et al., 2005). The clinical signs of atypical EAE are therefore distinctly different from those of classical disease. Classical EAE is characterised by increasing paralysis from the tail forwards, whereas atypical EAE presents with disturbances in balance and co-ordination. As mentioned earlier, no one model of EAE is able to recapitulate all the features of MS. Investigating atypical EAE could lead to insights into the mechanisms of the balance and co-ordination problems in MS sufferers.

A recent publication has shown that the transfer of polyclonal IFN-γ-deficient T cells into WT hosts resulted in the initiation of atypical EAE with the encephalitogenic T

cells infiltrating into the brainstem preferentially (Kroenke, Chensue and Segal, 2010). Interestingly, this induction of atypical EAE was dependent on IL-17 signalling because when IFN-γ-deficient cells were transferred into IL-17Rα-deficient mice, the clinical disease reverted to classical EAE, indicating that IL-17 signalling was required for the development of atypical EAE (Kroenke, Chensue and Segal, 2010). This also indicated, in an indirect manner that in the absence of both IFN-γ and IL-17 signalling, classical EAE is still induced suggesting that neither cytokine is required for EAE induction. In contrast, the induction of classical EAE was found to be dependent on GM-CSF production by $CD4^+$ T cells in the CNS (Kroenke, Chensue and Segal, 2010). Moreover, it has previously been shown that GM-CSFKO mice are resistant to disease induction (McQualter et al., 2001; Ponomarev et al., 2007).

Correlating with the role of IL-17 in the induction of atypical EAE, the constitutive production of IL-6 specifically within the cerebellum of mice resulted in the development of atypical EAE after MOG-immunisation (Quintana et al., 2009). The production of IL-6 within the cerebellum could lead to the generation of IL-17-producing Th17 response, which would result in the induction of atypical EAE due to the localised inflammation within the cerebellum as opposed to the spinal cord.

1.4 Trafficking of encephalitogenic T cells in to the CNS

The CNS was originally considered to be an immune privileged site. The blood brain barrier (BBB) acts as a physical barrier preventing the entry of lymphocytes into the CNS. In 1986 Wekerle and colleagues hypothesised that under normal physiological conditions activated lymphocytes are capable of entering the CNS to undertake immune-surveillance (Wekerle et al., 1986; Hickey, Hsu and Kimura, 1991). However, during inflammatory conditions, for example during MS and EAE, as well as during viral or bacterial infection, a large number of inflammatory cells gain access into the CNS across the BBB.

The CNS derives nutrients from two main sources –the blood vasculature that traverses the meninges and the parenchyma, and from the CSF. The blood brain barrier is formed by the vascular endothelium with the CNS parenchyma. The CSF is mainly made by a richly vascularized invagination of specialized secretory ependymal cells in the lateral, third and fourth cerebral ventricles, known as the choroid plexus. The CSF itself is contained within the sub-arachnoid space (SAS) which is created between the inner pia mater, an arachnoid membrane and the outer dura mater. The SAS allows circulation of the CSF around the brain and spinal cord. The epithelium of the choroid plexus constitutes the blood-CSF-barrier and the BBB is formed by the vascular endothelium with the CNS parenchyma (Engelhardt and Ransohoff, 2005; Abbott, Ronnback and Hansson, 2006). Tight junctions between the epithelial cells of the choroid plexus and between the vascular endothelial cells found within the CNS parenchyma forming physical barriers to entry into the CNS. These tight junctions restrict the flow of macromolecules from the blood into the CSF increasing the reliance on trans-cellular transport. Importantly, the tight junctions also prevent cellular egress. The capillary endothelial cells of the BBB are surrounded by a basal lamina, pericytes and astrocytic end-feet with microglia. Any physiological and pathological changes in the activity of the glial cell populations can weaken the BBB integrity and therefore its permeability. Pro-inflammatory cytokines for example IL-1β, IL-6 and TNF-α can be produced by these microglia

and contribute to increasing the permeability of the BBB (Prendergast and Anderton, 2009).

When a naïve T cell is activated, as mentioned in earlier sections, it differentiates into an effector T cell according to the local cytokine environment. During this process naïve T cells downregulate molecules required for entry into lymph nodes, for example, CD62L and CCR7 (von Andrian and Mempel, 2003; Klinger et al., 2009), and upregulate molecules involved in lymphocyte migration to non-lymphoid tissues, for example tissue-homing integrins, chemokine receptors and particular selectins. Each Th subset is believed to have a distinct pattern of expression of homing molecules. This differential expression of homing molecules would indicate differential abilities to enter inflamed tissues or differential abilities to cross the BBB or blood-CSF-barrier into the CNS. Human Th1 cells are known to express CCR5 and CXCR3 (Bonecchi et al., 1998; Sallusto et al., 1998) whereas Th2 cells express CCR3 and CCR4 (Sallusto, Mackay and Lanzavecchia, 1997; Sallusto et al., 1998). Th17 cells express CCR6 mainly and to a lesser extent CCR2 (Sato, Aranami and Yamamura, 2007; Liu and Rohowsky-Kochan, 2008; Singh et al., 2008). This correlates with chemokine receptor expression in mouse Th1, Th2 and Th17 polarised cells.

1.4.1 Routes of entry into the CNS

It is important to understand how lymphocytes are able to enter the CNS during an inflammatory response. There are thought to be three distinct entry routes into the CNS from the blood. These are 1) from the blood, via the choroid plexus into the CSF, or 2) from the blood, across the BBB or blood-spinal cord barrier into the parenchymal perivascular space, or 3) from the blood to the sub-arachnoid space (Ransohoff, Kivisakk and Kidd, 2003). It may be that entry via the CSF is required for immuno-surveillance of the CNS under normal physiological conditions, whereas entry to the parenchymal spaces of the brain can occur during localised inflammatory responses. Migration via the choroid plexus involves extravasation from the fenestrated capillary bed into the perivascular spaces. This is followed by crossing tight junctions of the ependymal epithelial layer. Under normal conditions, adhesion

molecules intercellular adhesion molecules (ICAM)-1, vascular cell adhesion molecule (VCAM)-1 and mucosal addressin cell adhesion (MAdCAM)-1 have been found to be expressed on the apical surface of the choroid plexus epithelial cells and not on the fenestrated capillaries of the choroid plexus (Wolburg et al., 1999; Kivisakk et al., 2003) and their expression was found to be increased during EAE.

The molecular mechanisms defining the entry of lymphocytes into the CNS either under normal conditions or under inflammatory conditions are still not well understood. The mechanisms involved in the entry of the initial pioneer lymphocytes into the CNS are very likely to be different from those involved in the entry once inflammation is established. Cell adhesion molecules, including selectins, integrins and chemokine receptors and their ligands are all involved in this process. Leukocyte trans-endothelial migration can be divided into four main steps, described below (as reviewed in (Prendergast and Anderton, 2009):

1. Low-affinity contact between the endothelium and the lymphocytes. This is mediated by selectins on the endothelial surface binding to the glycosylated ligands on the lymphocyte surface (Engelhardt, 2008). This low-affinity contact results in tethering of the lymphocyte to the endothelium, and facilitates their rolling along the endothelial cell surface.

2. Whilst rolling, the lymphocyte encounters chemokines secreted by endothelial cells. The activation of the corresponding chemokine receptors on the lymphocyte leads to the induction of G protein-linked intracellular signals. This results in the activation of the integrins on the surface of the lymphocyte. This activation alters the conformation of the integrin from the low affinity to the high affinity state.

3. The high affinity integrins are then able to interact with their appropriate ligands on the surface of the endothelium. This interaction provides firm adhesion or arrest of the lymphocyte on the endothelium surface.

4. The final step of lymphocyte trans-endothelial migration is the extravasation of the lymphocyte across the endothelial layer and into the perivascular space. This requires negotiating the tight junctions of the BBB or the blood-spinal cord barrier in order to enter the brain parenchyma or the spinal cord respectively (Engelhardt and Wolburg, 2004).

1.4.2 Selectins

Selectins are membrane glycoproteins with distal lectin-like domains. L-, P- and E-selectin constitute the family of Ca^{2+}-dependent lectins, which, as the names suggests, bind to and dissociate from, their ligands in a Ca^{2+}-dependent manner (Ley and Kansas, 2004). L-selectin (CD62L) is expressed on most circulating lymphocytes and is required for the entry of lymphocytes into the peripheral lymph nodes via high endothelial venules (Kanda et al., 2004; Miyasaka and Tanaka, 2004). L-selectin binds to glycosylation-dependent cell adhesion molecules-1 (GlyCAM-1) which is constitutively expressed on endothelial cells of the peripheral lymph nodes. CD62L also binds to MAdCAM-1. There is conflicting evidence as to whether CD62L is required for EAE. CD62L-deficient mice are protected from disease in myelin basic protein (MBP) specific systems (Archelos et al., 1998; Grewal et al., 2001). However, in the C57BL/6 or SJL mice, there was no role for CD62L in EAE, these mice being fully susceptible (Uboldi et al., 2008).

PSGL-1 is a 240 kDa homodimer that, if glycosylated, is capable of binding E-, P- and L-selectin (McEver, 1995; Moore, 1998). Therefore, despite PSGL-1 being expressed on all T lymphocytes not all of the different T cell subsets are capable of binding the selectins, depending on whether they are able to glycosylate the PSGL-1. The two enzymes that are required for the glycosylation and therefore expression of functional PSGL-1 on T lymphocytes are core 2 β-1.6-N-acetyl glycosaminyltransferase (C2GnT-I) and α-(1,3)-fucosyltransferase-VII (FucT-VII) (Smithson et al., 2001; Sperandio et al., 2001; Piccio et al., 2005; Deshpande, King and Segal, 2006). The glycosylation of PSGL-1 by these enzymes allows its binding to P-selectin in particular. Mice lacking either of these enzymes have impaired

binding of PSGL-1 to P-selectin, which leads to reduced rolling of lymphocytes on venules *in vivo* (Smithson et al., 2001; Sperandio et al., 2001).

The expression of functional PSGL-1 on Th1 cells and the binding to P-selectin is required for the migration of Th1 cells into the inflamed skin *in vivo* (Austrup et al., 1997; Borges et al., 1997). In contrast despite Th2 cells also expressing PSGL-1 on their surface it was not necessary for the migration of Th2 cells into the inflamed skin, highlighting a particular requirement of functional PSGL-1 on Th1 cells specifically, for their entry into the inflamed skin (Borges et al., 1997).

The requirement of P-selectin and PSGL-1 for entry into the CNS remains controversial. PSGL-1-deficient C57BL/6 mice, or C57BL/6 mice treated with a blocking antibody against PSGL-1, do not have a significant difference in disease induction or severity compared to wild-type controls (Engelhardt et al., 2005; Osmers, Bullard and Barnum, 2005). Another study however does suggest a role for the P-selectin/PSGL-1 interaction for the entry of T cells into the CNS. IL-12p70 has been found to increase the expression of C2GnT-I (Deshpande, King and Segal, 2006), leading to the increase in expression of functional PSGL-1. The pre-treatment of IL-12-stimulated myelin-reactive $CD4^+$ T cells with a blocking antibody against PSGL-1 prior to adoptive transfer led to significantly reduced onset, incidence and severity of EAE (Deshpande, King and Segal, 2006). Therefore, depending on the model, PSGL-1 may indeed have a role to play in the entry of encephalitogenic T cells into the CNS across the BBB or the B-CSF-B as summarised in Figure 1.6 at the end of this section.

1.4.3 Chemokines and chemokine receptors

Chemokines are small (8-10 kDa) proteins that fall into four main families of chemokines categorised on their structure and relative location of their cysteine residues. The largest family is the CC chemokines in which the first two cysteine residues are adjacent to each other. The three remaining families include the CXC chemokines, the CX_3C chemokines and the XC family of chemokines, which have differing numbers of amino acids between the cysteine residues.

Chemokines function by binding their corresponding seven-transmembrane-domain G-protein-couple receptors (Charo and Ransohoff, 2006) which leads to a signalling cascade. There are two main mechanisms by which chemokine binding can influence the migration of leukocytes across the BBB. Firstly, as mentioned earlier, chemokines immobilised on the surface of the endothelium binding to their respective receptor on the surface of the lymphocyte results in the activation of surface integrins on the lymphocyte, leading to the firm adhesion of the leukocyte. CCL19 (macrophage inflammatory protein (MIP)-3β), CCL20 (MIP-3α) and CCL2 (monocyte chemotactic protein (MCP)-1) have been shown to induce adhesion via activation of LFA-1 binding to ICAM-1 (Campbell et al., 1998; Gerszten et al., 1999).

The second mechanism by which chemokines can influence migration is by mediating the locomotion of the leukocytes along the endothelium to their nearest endothelial tight-junction. As the leukocyte migrates through the tight junction it is thought to extend chemokine receptor-rich processes and these seek chemokines in the abluminal space (Schreiber et al., 2007). This chemokine dependent mechanism facilitates their movement through the tight junction and into the perivascular space. Due to the different roles of chemokines and their receptors, dissecting their specificity and function at each stage is challenging.

Due to the original hypothesis that MS and EAE were Th1-mediated diseases, numerous studies investigated the requirements for CCR5 and CXCR3 in entry into the CNS, both of these chemokine receptors being Th1-associated. The ligands of CXCR3 and CCR5 the chemokines IP-10 (CXCL10), MIP-1α (CCL3) and RANTES (regulated upon activation, normal T cell expressed and secreted (CCL5)) were found to be upregulated in the CSF of MS patients during episodes and also within MS lesions (Balashov et al., 1999; Sorensen et al., 1999). In addition to this $CCR5^+$ and $CXCR3^+$ T cells were increased in the blood and CFS of MS patients (Sorensen et al., 1999; Teleshova et al., 2002).

As mentioned above, $CCR5^+$ T cells are found to be upregulated in MS patients. However the requirement of CCR5 for EAE induction is still controversial. C57BL/6 CCR5-deficient mice, or MIP-1α-deficient (one of the ligands of CCR5) mice are susceptible to MOG-induced EAE (Tran, Kuziel and Owens, 2000). These mice showed no differences in the kinetics or severity of disease compared to the wild-type mice, or in the infiltration of lymphocytes into the CNS. In contrast, it has been shown that in the model experimental autoimmune uveitis (EAU) CCR5 is required for the entry of Th1 cells into the target organ, the eye (Crane et al., 2006).

CCR6 and its ligand are now considered to be Th17-associated migration molecules. Both CCR6 and its ligand have been found to be upregulated in the spinal cord during EAE (Fife et al., 2001). Recently, since the emergence of the Th17 lineage, more studies have been done on the requirements of CCR6 for CNS inflammation, often with conflicting results. Two studies showed total protection from EAE in the absence of CCR6 (Liston et al., 2009; Reboldi et al., 2009) whilst a third study reported only delayed onset (Yamazaki et al., 2008). The mechanisms underlying the observed effects also conflicted with two reports of impaired migration of the Th17 cells into the CNS (Yamazaki et al., 2008; Reboldi et al., 2009) and another implicated altered T cell priming in the lymph nodes (Liston et al., 2009). Interestingly one report implicated the CCR6/CCL20 interaction specifically within the choroid plexus as a key check-point in Th17 cell entry into the CNS. Small numbers of $CCR6^+$ cells were found to restore susceptibility to EAE in CCR6-deficient mice. Intense expression of CCL20 was observed specifically at the choroid plexus. Greater numbers of T cells could be found in the choroid plexus of CCR6-deficient mice than in wild-type mice primed for EAE (Reboldi et al., 2009), suggesting that an intact CCR6/CCL20 interaction is essential, not for extravasation, but to allow the accumulating pathogenic T cells to cross the tight junctions of the ependymal layer that constitute the blood-CSF-barrier as shown in the summary diagram, Figure 1.6 at the end of this section.

Treg have also been shown to express CCR6 (Kleinewietfeld et al., 2005; Lim et al., 2008; Yamazaki et al., 2008). If CCR6 expression is indeed required for the

infiltration of T cells into the CNS, this suggests that both effector Th17 cells and regulatory T cells and perhaps even effector Th1 cells if they are found to express CCR6, are able to infiltrate the CNS at the same time and route. When targeting particular cell types for therapeutics and the prevention of inflammation in the CNS, this would pose a problem, especially if blocking a $CCR6^+$ inflammatory T cell from infiltrating the CNS would also prevent $CCR6^+$ Tregs from infiltrating.

1.4.4 Integrins

Integrins mediate the firm adhesion of leukocytes to the surface of the endothelium. Structurally, integrins are heterodimeric proteins containing α and β chains. There are 18 different α subunits and 8 β subunits in vertebrates, and together these form 24 known αβ pairs. Approximately 10 members of the integrin family of the β2, β7, and β1 subfamilies are expressed by immune cells including monocytes, macrophages, T lymphocytes, dendritic cells and neutrophils. The β2 and β7 subfamilies are expressed on leukocytes whereas the β1 subfamily of integrins is expressed on a wide variety of cells (Reviewed in (Luo, Carman and Springer, 2007)).

Integrin signalling involves two forms of signalling: 'inside-out' and 'outside-in'. 'Inside-out' signalling results in a conformational change of the integrin which leads to increased affinity for ligand binding as well as clustering of integrins in the membrane. In contrast, 'outside-in' signalling refers to the signalling events occurring after the clustering of integrins. Circulating lymphocytes maintain their integrins in a non-adhesive conformation which does not support ligand binding. On integrin activation, this conformation changes to an extended integrin conformation due to unfolding of the ectodomain of the receptor. This extended conformation facilitates clustering of integrins on the cell membrane and high affinity binding of the ligand to the receptor. The family of integrins include LFA-1 (αLβ2) which binds ligands ICAMs 1-5; α4β1 which binds VCAM-1 as well as α4β7 (reviewed in (Abram and Lowell, 2009)). Defective integrin expression results in human conditions known as leukocyte adhesion deficiencies (LAD) (Abram and Lowell, 2009).

It has been shown that ICAM-1 is expressed on the endothelium surrounding the CNS lesions both in patients with MS and in mice with EAE suggesting a role in entry of the inflammatory T cells into the CNS (Raine et al., 1990; Sobel, Mitchell and Fondren, 1990; O'Neill et al., 1991). Numerous studies have implied a role for the LFA-1/ICAM-1 interaction in lymphocyte adhesion and migration across the blood brain barrier into the CNS (Archelos et al., 1993; Steffen, Butcher and Engelhardt, 1994; Laschinger, Vajkoczy and Engelhardt, 2002; Lyck et al., 2003; Schenkel, Mamdouh and Muller, 2004). Data suggests that the LFA-1/ICAM-1 interaction is required for the movement of the adhered cell across the endothelium to the nearest tight junction, as opposed to being required for the adhesion of the lymphocyte to the activated endothelium. This is consistent with *in vitro* studies that have shown that cells lacking ICAM-1 are still capable of supporting adhesion of T cells to the activated endothelium however are unable to support transmigration across the endothelium (Lyck et al., 2003).

Complementary to this, the α4β1/VCAM-1 interaction has been shown to be required for the firm adherence of lymphocytes to the endothelial surface and not for the transmigration across the endothelium (Laschinger and Engelhardt, 2000). Although VCAM-1 binds both α4β1 and α4β7, only α4β1 is required for the migration of T cells across the blood brain barrier during EAE (Engelhardt et al., 1998). MAdCAM-1, the ligand for α4β7 integrin, and ICAM-1 and VCAM-1 are all upregulated on the apical surface of the choroids plexus epithelial cells during EAE (Steffen et al., 1996; Engelhardt, Wolburg-Buchholz and Wolburg, 2001). However the blockade of MAdCAM-1, the ligand for α4β7, has been shown to prevent the development of EAE (Kanwar et al., 2000). VCAM-1, ICAM-1 and MAdCAM-1 have each been shown to have a role in T cell migration across the blood brain barrier. Combination treatments with anti-MAdCAM-1, VCAM-1 and ICAM-1 were trialled and faster remission from disease was observed, however the same effect was not found with advanced EAE (Kanwar et al., 2000). Activated leukocyte cell adhesion molecule (ALCAM; CD166) another integrin, is upregulated on the endothelium of the blood brain barrier in both EAE and MS and its blockade results

in reduced severity of disease due to the inability of $CD4^+$ lymphocytes and monocytes to enter the CNS during EAE (Cayrol et al., 2008).

In summary, the obvious requirement of integrins in the migration of lymphocytes into the CNS makes them an attractive target for therapeutics to prevent the entry of inflammatory T cells into the CNS, some of which have already been successfully exploited (as discussed in the MS therapeutics section).

Once T cells have penetrated the endothelial surface in to the perivascular space, they still need to infiltrate the brain parenchyma via the basement membrane which consists of extracellular matrix proteins (for example laminins, collagen type IV, nidogens and heparin sulphate proteoglycans) (Hallmann et al., 2005). Chemokines and matrix metalloproteinases (MMPs) are required for this process (as reviewed in (Prendergast and Anderton, 2009).

Targeting the infiltration of inflammatory T cells into the CNS to prevent inflammation is an attractive therapeutic target, especially due to the vast number of molecules involved in the process. The potential for this route of therapeutics has already been proven by the success of Natalizumab in treatment of MS (Miller et al., 2003). The challenge is finding a target that is non-redundant, yet specific enough to target particular subsets of lymphocytes that infiltrate the CNS to cause autoimmune inflammation. This must also not compromise essential immune surveillance.

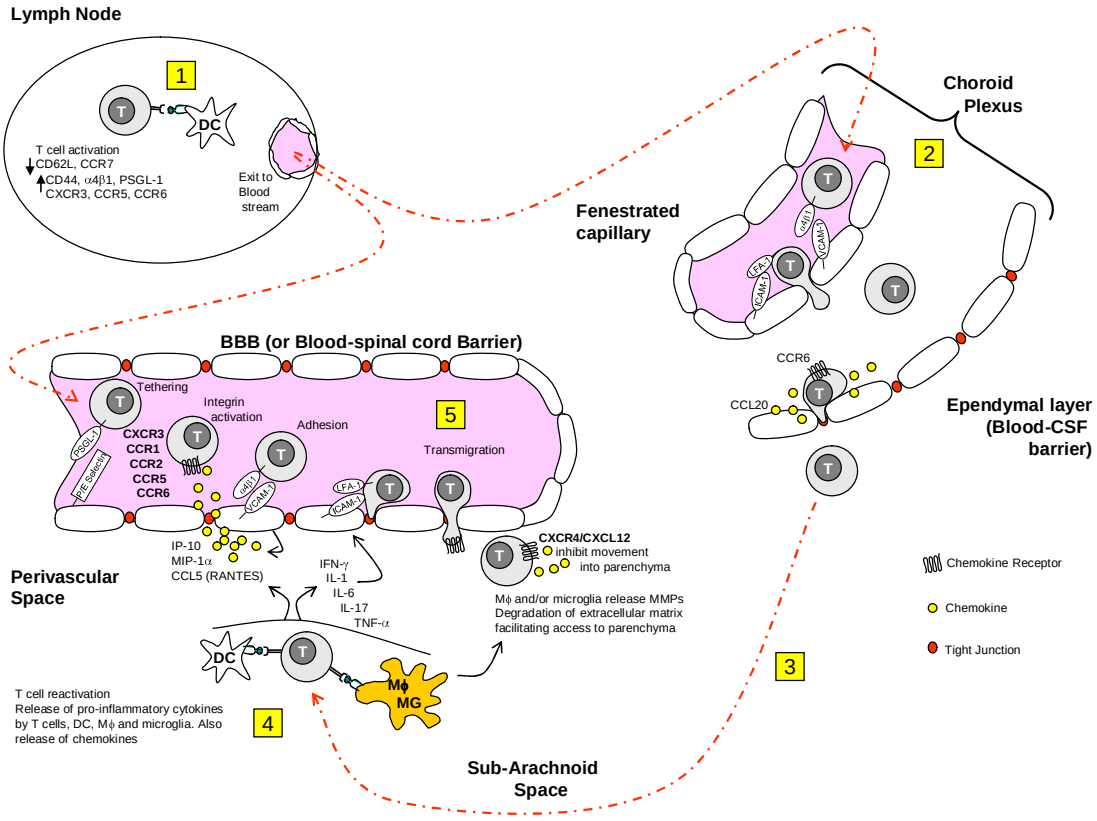

Figure 1.7 Summary of requirements for entry of T cells into the CNS via the BBB and the blood-CSF-barrier to induce CNS autoimmune disease.

1) Myelin responsive T cells are activated in the lymph node in the presence of their cognate antigen. The naïve T cells downregulate lymph node homing molecules and express integrins and chemokine receptors indicative of their Th phenotype. These activated T cells then exit the lymph node and enter the circulation; 2) T cells reach the choroid plexus and cross the fenestrated capillaries. This is mediated by expression of CCL20 and CCR6; 3) The T cells migrate via the CSF through the sub-arachnoid space and into the brain parenchyma or spinal cord parenchyma; 4) T cells are re-activated and produce inflammatory cytokines. The inflammatory conditions lead to the activation of the local endothelium and expression of adhesion molecules and the release of chemokines by immune cells and the stromal/endothelial cells; 5) Circulating T cells with the appropriate homing molecules localise on the CNS endothelium and extravasate into the perivascular space. MMPs facilitate the process of migrating across the perivascular space and into the brain parenchyma. Figure taken from Prendergast and Anderton, 2009 with permission from EMID-DT, Bentham Science Publishers.

1.5 Hypothesis

The central hypothesis for this thesis was based on the following observations:

- Treg are required for the resolution of actively-induced EAE.
- Passive transfer of Th1 cells provides resolving/monophasic EAE with Treg accumulation evident in the CNS.
- CNS derived Treg can suppress Th1 effector function (IFN-γ production) *in vitro*, but not Th17 function (IL-17 production).

These observations led to the following hypothesis:

'Myelin-responsive Th17 cells produce chronic EAE due to their resistance to Treg mediated suppression, whereas their Th1 counterparts provide only acute EAE.'

1.6 Aims

The aims of this PhD project are three-fold:

- To establish rigorous Th1 and Th17 polarisation and culture conditions to generate 'clean' populations of both IFN-γ-producing Th1 and IL-17-producing Th17 cells devoid of any contaminating cells.
- To compare EAE driven by myelin reactive Th1 and Th17 cells using the passive transfer system.
- To investigate the molecular mechanisms underlying any differences seen in pathogenic activity of Th1 versus Th17 cells.

2 Materials and Methods

2.1 Mice

C57BL/6 (H-2^b background; CD45.2/CD90.2; CD45.1/CD90.2, or CD45.2/CD90.1), B10.PL (H-2^u background), Tg4 (Liu et al., 1995) (CD45.1 or CD90.1) and H-2^b IFN-γKO (Dalton et al., 1993) mice were bred under specific pathogen-free conditions at the University of Edinburgh (Edinburgh, U.K.). Experiments received University of Edinburgh ethical approval and were performed under U.K. legislation. Tg4 CD45.1 IFN-γKO mice were generated at the University of Edinburgh (Edinburgh, U.K.). This was done by crossing the IFN-γKO mice with Tg4 CD45.1 mice and back-crossing for ten generations. Before ten generations was reached cells from Tg4 CD45.1 IFN-γKO mice were transferred into B10.PL x C57BL/6 hosts. All mice were age and sex matched for experiments and were used between the ages of 6-10 weeks.

2.2 Peptides

Myelin oligodendrocyte glycoprotein peptide 35-55 ($pMOG_{35-55}$ MEVGWYRSPFSRVVHLYRNGK) and myelin basic protein acetylated peptide (MBP Ac1-9; Ac-ASQKRPSQR) were synthesised by the Advanced Biotechnology Centre, Imperial College London (London, U. K.).

2.3 General Reagents

2.3.1 Wash Buffer

RPMI 1640 medium containing 25 mM Hepes (Gibco, Invitrogen, Life Technologies, Paisley, UK) supplemented with 2 mM L-Glutamine (Gibco), 100 U/ml Penicillin (Gibco), 100 μg/ml Streptomycin (Gibco) and 50 μM 2-β-Mercaptoethanol (Gibco).

2.3.2 RPMI-10%

RPMI 1640 medium containing 25 mM Hepes (Gibco), supplemented with 10 % heat-inactivated fetal calf serum (FCS; Sigma), 2 mM L-Glutamine (Gibco), 100 U/ml Penicillin (Gibco), 100 µg/ml Streptomycin (Gibco) and 50 µM 2-β-Mercaptoethanol (Gibco).

2.3.3 MACS Buffer

Hanks Balanced Salt Solution (Gibco) supplemented with 2 % heat-inactivated FCS (Sigma).

2.3.4 FACS Buffer

PBS supplemented with 2 % heat-inactivated FCS (Sigma) and 0.1 % sodium azide (Sigma).

2.4 T cell isolation, purification and sorting

2.4.1 Single cell suspensions from spleen and lymph nodes

Spleen and lymph nodes were harvested from mice and mashed through gauze (Sefar) to obtain a single cell suspension. Red blood cells (RBC) were lysed by treatment with 2 ml Red Blood Cell Lysis Buffer (Sigma) for 2 minutes at room temperature and then washed with RPMI and the number of live cells determined by trypan blue (Sigma) exclusion. Samples were then used for flow cytometry analysis, or for cell sorting as outlined below.

2.4.2 $CD4^+$ Sorting by Magnetic cell sorting

After RBC lysis and counting, cells were resuspended in MACS buffer and counted. Cells were then resuspended in 45 µl MACS Buffer per 10^7 cells and 5 µl CD4 (L3T4) Microbeads (Miltenyi) added per 10^7 cells. Cells were incubated at 4 °C for 20 minutes with frequent mixing, and then washed in MACS buffer and resuspended in 3 ml MACS Buffer. The $CD4^+$ cells were then positively selected using an MS or

LS column (Miltenyi) using the manufacturers protocol: After preparing the column by rinsing through with 3 ml MACS Buffer, the sample was applied to the column, followed by 3x3ml MACS Buffer washes to remove the unlabelled cells. The $CD4^+$ T cells were then flushed out by removing the column from the magnet and putting it into an appropriate collection tube and firmly flushing through 5 ml MACS Buffer using the plunger. The positive fraction of cells were then counted to determine the yield and washed in MACS Buffer.

2.4.3 Naïve T cell sorting by FACS

Naïve Tg4 $CD45.1^+$ or Tg4 $CD90.1^+$ T cells were sorted from splenocytes and lymph node cells to culture and polarise in the presence of irradiated splenocytes. After CD4 sorting by magnetic columns (above) the cells were stained with anti-CD4-APC, anti-CD62L-FITC and anti-CD25-PE in MACS Buffer at 4 °C for 20 minutes. Cells were then washed in MACS Buffer, and resuspended in ~3 ml MACS Buffer (at ~$1x10^7$ cells/ml). The naïve cells were then sorted on the FACS Aria for the $CD4^+$ $CD62L^{hi}$ $CD25^-$ population.

2.4.4 Isolation of CNS-infiltrating mononuclear cells

Mice were sacrificed by CO_2 asphyxiation and perfused through the eye or through the left ventricle of the heart with 10 ml cold PBS. The brain was removed by dissection and the spinal cord by intrathecal hydrostatic pressure. Tissues were cut up into small pieces using scissors and disrupted using a 1 ml syringe, and subsequently digested with 300 µl of 2.5 mg/ml collagenase (Lorne Laboratories) and 100 µl of 10 mg/ml deoxyribonuclease (Sigma) at 37 °C for 40 minutes. Cells were then washed once with RPMI. The mononuclear cells were recovered from the interface of a 30:70 discontinuous Percoll (GE Healthcare) gradient after centrifugation for 20 minutes at 850 x g without brake, and washed in RPMI.

2.5 *In vivo* manipulations

2.5.1 Immunisations

Mice were immunised with 100 μg $pMOG_{35-55}$, or 100 μg MBP (Ac1-9), emulsified in Complete Freund's Adjuvant (CFA) containing 50 μg heat-killed *Mycobacterium tuberculosis* H37Ra (Sigma) at a final volume of 100 μl; 50 μl injected sub-cutaneously (s.c.) into each hind leg.

2.5.2 Induction of Passive EAE

Pre-activated myelin specific cells (either *in vivo* primed and *in vitro* polarised polyclonal Th1/Th17 cells, or, transgenic Tg4 Th1/Th17 cells) were harvested and washed in serum free PBS (Gibco) and counted. $4x10^6$ blasts were transferred per host (unless stated otherwise) by intravenous (i.v.) injection in a volume of 200 μl in PBS (Gibco). Host mice also received 200 ng Pertussis Toxin (P.tx; Health Protection Agency, Dorset, UK) in 500 μl PBS by intra-peritoneal (i.p.) injection. Clinical signs of EAE were assessed daily from day 6 post-transfer using the system/s described below in sections 2.5.4 and 2.5.5.

2.5.3 Induction of Active EAE

Mice were immunised s.c. with 100 μg $pMOG_{35-55}$, or 100 μg MBP (Ac1-9), emulsified in Complete Freund's Adjuvant (CFA) containing 50 μg heat-killed *Mycobacterium tuberculosis* H37Ra, at a final volume of 100 μl with 50 μl in each hind-leg . Mice also received 200 ng P.tx i.p. in 500 μl PBS on day 0 and day 2 post-immunisation. Clinical signs of EAE were assessed daily from day 6 post-immunisation using the system described below in sections 2.5.4 and 2.5.5.

2.5.4 Scoring of Classical EAE

0, no clinical signs, healthy; **1,** flaccid tail; **2,** impaired righting reflex and/or walk; **3,** partial hind limb paralysis; **4,** total hind limb paralysis; **5,** hind limb paralysis with any partial front limb paralysis; **6,** moribund or dead.

2.5.5 Scoring of atypical EAE

0; no clinical signs; **1,** head tilted slightly/hunched appearance; **2,** head turning more pronounced/ ataxia/ scruffy coat; **3,** body tilted and inability to walk in a straight line; **4, l**aying on side or involuntary and continuous rotation; **5,** moribund. (Adapted from (Stromnes et al., 2008)).

2.5.6 *In vivo* Administration of Tak-779

Tak779 was kindly supplied as a free gift from the NIH AIDS Research and Reference Reagent program (catalogue number 4983). Tak-779 (*N*,*N*dimethyl-*N*-[4-[[[2-(4-methylphenyl)-6,7-dihydro-5*H*-benzocyclohepten-8-yl]carbonyl]amino]benzyl]tetrahydro-2*H*pyran-4-aminium chloride, M_r 531.13) was resuspended in PBS to 5 mg/ml, aliquoted and stored at -20 °C. For administration, Tak779 was further diluted to 250 μg/ml and each host mouse received 200 μl (i.e. 50 μg Tak779) i.v. every other day as stated in the experiment scheme.

2.5.7 Administration of anti-CD62L (Mel-14)

Mel-14 was obtained from Bioxcel. The stock was diluted to 500 μg/ml in PBS and each mouse received 200 μl (i.e. 100 μg Mel-14) i.v. every other day as stated in the experiment scheme.

2.5.8 Administration of anti-CD162/PSGL-1 (4RA10)

Anti-CD162/PSGL-1 (4RA10) and its isotype control, IgG1, were obtained from BD Pharmingen. For the *in vitro* pre-treatment of cells with anti-PSGL-1, *in vitro* polarised Tg4 Th1 cells were harvested and treated with 10 μg/ml anti-CD162, or 10 μg/ml IgG1 isotype control, for 1 hour on ice whilst at a density of 5-10x10^6 cells/ml. Cells were then washed in PBS to remove any unbound antibody and resuspended in PBS for transfer into host mice. For the *in vivo* treatment with anti-PSGL-1, the stock antibody (1 mg/ml) was further diluted to 125 μg/ml in PBS, and 200 μl administered per mouse i.v. to give a dose of 25 μg per mouse.

2.6 Cell culture and T cell polarisations

2.6.1 TCR Transgenic system

Tg4 CD45.1, Tg4 CD90.1 or Tg4 CD45.1 IFN-γKO spleens and lymph nodes were taken. Tissues were disrupted to form a single cell suspension and treated with RBC lysis buffer (Sigma), MACS sorted for the $CD4^+$ fraction and subsequently sorted by FACS for the naïve $CD4^+$ T cells i.e. $CD4^+$ $CD62L^{hi}$ $CD25^-$ as described in sections 2.4.1-2.4.3. Naïve T cells were cultured in a 1:5 ratio with irradiated splenocytes from B10.PL or B10PLxC57BL/6 mice as antigen presenting cells (APCs) for 72 hours with 10 μg/ml MBP (Ac1-9). When whole splenocytes were used for polarisations, Tg4 CD45.1 or Tg4 CD90.1 splenocytes and lymph node cells were treated as above and then cultured at a concentration of $4x10^6$ cells/ml for 72 hours with 10 μg/ml MBP (Ac1-9) and relevant polarising cytokines as outlined below in section 2.6.1.3.

2.6.2 Polyclonal system

C57BL/6, CD45.1 or CD90.1 mice were immunised s.c. with 100 μg $pMOG_{35-55}$ with CFA. Ten days later the draining lymph nodes were excised and the cells recovered and cultured at a concentration of $4x10^6$ cells/ml, with 10 μg/ml $pMOG_{35-55}$ and relevant polarising cytokines as outlined below in section 2.6.1.3.

2.6.3 *In vitro* polarisation towards Th1, Th17 and Th2 phenotype

To achieve a Th1 polarisation, cells were cultured with 25 ng/ml rIL-12 (R&D Systems), 25 ng/ml rIL-18 (MBL) and 0.5 ng/ml rIL-2 (R&D Systems) for 72 hours. The rIL-2 concentration was increased to 2.5 ng/ml for the final 24 hours of culture. For a Th17 polarisation, cells were cultured with 20 ng/ml rIL-6, 20 ng/ml rIL-23 and 3 ng/ml rTGF-β (all R&D Systems) for 72 hours. For a Th2 polarisation, cells were cultured with 4 ng/ml rIL-4 (Peprotech), 5 μg/ml anti-IL-12 (Bioxcell), 5μg/ml anti-IFN-γ (Bioxcell) and 40 U/ml rIL-2 for 5 days.

2.6.4 Re-stimulation of T cells for analysis of peptide specific cytokine production

For cells being re-stimulated in the presence/absence of peptide for subsequent intracellular cytokine staining, cells were cultured at $8x10^6$ cell/ml in the presence/absence of 20 μg/ml peptide ($pMOG_{35-55}$ or MBP (Ac1-9)) over-night. The following day, Brefeldin A (eBioscience) was added at a 1:1000 final dilution for 4-6 hours prior to intracellular cytokine staining.

2.7 Fluorescence Assisted Cell Sorting Analysis

2.7.1 Antibodies

Antibodies used were all from eBioscience unless stated otherwise. See Table 2.1 for a list of all the antibodies, conjugates, clones and dilutions used.

2.7.2 Surface staining

Cells were stained in 25 μl FACS buffer using the antibodies outlined in Table 2.1 (from eBioscience unless stated) for 15 minutes at 4 °C. Afterwards cells were washed in FACS buffer and resuspended in either 200 μl FACS buffer and collected immediately, or in 200 μl 1% paraformaldehyde (PFA; Sigma) and stored at 4 °C and collected as soon as possible.

2.7.3 Intracellular cytokine staining

Cells were incubated with Brefeldin A (1:1000 dilution of stock; eBioscience) for the final 4-6 hours of culture. For maximal stimulation and cytokine production, cells were also re-stimulated for these 4-6 hours with 50 ng/ml phorbol myristate acetate (PMA) and 1 μg/ml Ionomycin (Sigma). Cells were washed in FACS buffer and resuspended in 50 μl permeabilisation buffer (FACS Buffer containing 0.1% saponin; Sigma) containing the appropriate anti-cytokine antibody or relevant isotype control and were stained for 30–40 minutes at room temperature in the dark. Cells were then washed in FACS Buffer and stained for the cell surface markers of interest in 25 μl FACS Buffer for 15 minutes at 4 °C in the dark and subsequently washed in FACS Buffer and resuspended in 200 μl FACS buffer or in 200 μl 1 % PFA.

2.7.4 FoxP3/T-bet and RORγt staining

Cells were stained for cell surface markers of interest as explained in section 2.7.2 first and then washed in FACS Buffer. Subsequent staining for FoxP3, T-bet or RORγt was done using the eBioscience FoxP3 staining buffer set, as per the manufacturer's protocol. Cells were incubated in 400 µl Fixation/Permeabilisation Buffer (eBioscience) for at least 1 hour, or overnight, and subsequently washed in FACS Buffer to permeabilise the cells. Cells were then stained in 50 µl 1x Permeabilisation Buffer (eBioscience) containing the anti-FoxP3/T-bet/RORγt antibody or appropriate isotype controls for 30 minutes at 4 °C in the dark. After this cells were washed in FACS Buffer and resuspended in 200 µl FACS Buffer or 200 µl 1% PFA and collected as soon as possible.

2.7.5 Intracellular cytokine staining in conjunction with FoxP3/T-bet or RORγt staining

When performing intracellular cytokine staining together with intracellular staining for transcription factors, cells were first stained for surface markers of interest as above in section 2.7.2. Then cells were washed in FACS buffer and resuspended in 400 µl Fixation/Permeabilisation Buffer (eBioscience) for at least 1 hour, or overnight. Cells were then washed in FACS Buffer and stained with the appropriate anti-cytokine and anti-transcription factor antibodies in 50 µl 1x Permeabilisation Buffer (eBioscience) for 30 minutes at 4 °C in the dark. Cells were then washed in FACS Buffer and resuspended in 200 µl FACS buffer or 1% PFA prior to collection.

2.8 Flow cytometry data analysis

For all intracellular staining flow cytometry analysis, gates were determined from the isotype control for each sample. Representative gating strategies are highlighted in the relevant appendices. All flow cytometry data was collected on the LSR II flow cytometer, LSR Fortessa or Canto (all BD Biosciences) and data analysed using FlowJo Software (Treestar version 3.2.1). FACS was performed using the FACS Aria (BD Biosciences).

2.9 IL-17 Secretion Assay –Cell Enrichment and Detection Kit Miltenyi Biotec

Assay specific reagents:

Buffer: phosphate buffered saline pH 7.2 (Sigma) containing 0.5% bovine serum albumin (BSA) (Sigma) and 2mM EDTA (Sigma). Filter sterilised and allowed to cool to 4 °C.

Culture Medium: RPMI 1640 containing 5% FCS.

Method

1) *In vitro* re-stimulation: Three day *in vitro* polarised cells were re-stimulated with 10 μg/ml MBP (Ac1-9) for 3 hours at 37 °C. Negative controls were un-stimulated cells. Cells were then collected by rinsing out of the plate with cold buffer to ensure collection of all cells. Viable cells were counted using trypan blue exclusion and $2x10^7$ live cells were used per 50 ml tube.

2) Labelling cells with IL-17 Catch Reagent: Cells were first labelled with IL-17 catch reagent. Cells were washed with 10 ml cold buffer, centrifuged at 300xg for 5 minutes at 2-8 °C and the supernatant removed. The cell pellet was resuspended in 80 μl of cold medium per 10^7 total cells and 20 μl IL-17 Catch Reagent added per 10^7 total cells, mixed and incubated for 5 minutes on ice.

3) IL-17 secretion period: As greater than 1% IL-17-secreting cells was a possibility, and to avoid non-specific staining, a large volume of pre-warmed (37 °C) medium was added to each tube (i.e. 30 ml) for the IL-17 secretion period. Cells were incubated in the closed tubes for 45 minutes at 37 °C with regular turnings of the tubes to prevent settling of the cells. The tube turning prevented cell to cell contact in the tube, to avoid cross contamination with cytokines.

4) Labelling of cells with IL-17 Detection Antibody (PE): The tube was then placed on ice, topped up with cold buffer and centrifuged at 300xg for 5 minutes at 2-8 °C and the supernatant removed completely. The cell pellet was then resuspended in 80 μl cold buffer per 10^7 total cells and 20 μl of IL-17 Detection Antibody (PE) added per 10^7 total cells. At this stage, cells were also stained with surface staining antibodies (i.e. 10 μl anti-CD4-APC). The cells were mixed well and incubated for 10 minutes on ice. Cells were then washed by adding 10 ml cold buffer

per 10^7 total cells and centrifuged at 300xg for 10 minutes at 2-8 °C and the supernatant removed completely.

5) Magnetic labelling: The cell pellet was then resuspended in 80 µl of cold buffer per 10^7 total cells and 20 µl of Anti-PE MicroBeads added per 10^7 total cells. The cells were mixed well and incubated for 15 minutes in the fridge (2-8 °C). After incubation, the cells were washed by adding 10 ml cold buffer per 10^7 total cells and centrifuged at 300xg for 10 minutes at 2-8 °C and the supernatant removed. The cell pellet was then resuspended in 500 µl of cold buffer (and a small aliquot removed at this stage for flow cytometric analysis of the pre-sort sample).

6) Magnetic separation: An MS column was placed in the magnetic field of a MACS separator and rinsed with 500 µl of buffer and the flow through discarded. The cell suspension was applied to the column and the unlabeled cells collected in a fresh tube. Three separate 500 µl washes were applied to the column and the effluent collected as the unlabelled fraction of cells. The column was then removed from the separator and placed in a collection tube, 1 ml of buffer applied to the column and the magnetically labelled cells flushed out by firmly pushing the plunger into the column. As the negative unlabelled cells were the cells of interest in this case, these cells were reapplied to a fresh column, and three more 500 µl washes performed and the unlabelled fraction collected. This second column sort was performed to increase the purity of the cell population obtained.

Analysis: a small fraction of the negative and positive fractions were taken for flow cytometric analysis of the cells post sort.

2.10 Cytokine quantification by ELISA

For the analysis of IFN-γ and IL-17 cytokine production an enzyme-linked immunosorbent assay (ELISA) was used. MaxiSorp microtiter plates (Nunc International) were coated with 50 µl/well of the relevant cytokine-capture antibody diluted in 1x bicarbonate buffer (10x stock: 6.36 g Na_2CO_3 (BDH) + 11.72 g $NaHCO_3$ (BDH) in 400 ml dH_2O, pH 9.6) i.e. 2 µg/ml anti-IFN-γ (clone R4-6A2); 0.5 µg/ml anti-IL-17 (clone TC11-18H10) (all BD Pharmingen), and incubated at 4 °C overnight. The following day, plates were washed twice with PBS 0.1 % Tween (PBS-Tween; Sigma) and then blocked for 1 hour at 37 °C with 200 µl/well PBS/1 %

BSA (PBS/BSA; Sigma). Plates were then washed twice with PBS-Tween. A ten point standard curve of doubling dilutions across the plate of the relevant cytokine was then set up (100 μl/well in duplicate) with the following top final concentrations: anti-IFN-γ 100 ng/ml and anti-IL-17 10 ng/ml (anti-IFN-γ standard from BD Pharmingen; anti-IL-17 standard from R&D). 4 blank wells with 100 μl/well PBS/BSA were also set up. Duplicate samples of relevant cell supernatant (100 μl/well) were added and the plates incubated for 2 hours at room temperature. Plates were then washed four times with PBS-Tween. The detection antibody was then added (100 μl/well) at the following final concentrations: anti-IFN-γ (clone XMG.12) 0.5 μg/ml and anti-IL-17 (clone TC11-8H4.1) 0.25 μg/ml diluted in PBS/BSA (all from BD Pharmingen). Plates were incubated at room temperature for 30 minutes and subsequently washed six times with PBS-Tween. The ELISA was then developed by adding 100 μl/well 3,3',5,5'-Tetramethylbenzidine (TMB) solution (TMB solution: 100 μl 10 mg/ml TMB in DMSO (both Sigma), 9.9 ml phosphate-citrate buffer (25.7 ml 0.2 M Na_2HPO_4 (BDH) + 24.3 ml 0.1 ml citrate (anhydrous) (Fluka) made up to 100 ml, pH 5)), 3 μl H_2O_2 (Sigma)). Reactions were then stopped by the addition of 100 μl 2 M H_2SO_4 (BDH) and the plate read at 450 nm using a Multiskan plate reader (Labsystems,UK). Data was then analysed using Prism software.

2.11 Molecular Biology Protocols

2.11.1 RNA Extraction by Chloroform/Isopropanol Method

For the analysis of mRNA expression levels in cells derived from immunised mice, cells were washed in cold PBS, centrifuged at 12,000xg for 5 minutes. The PBS was then carefully removed and the cell pellet resuspended thoroughly in 1 ml Qiazol Lysis Reagent (Qiagen) and stored at -80 °C. To extract the RNA from these samples the chloroform/isopropanol method of RNA extraction was used. Qiazol samples were thawed slowly on ice. Once fully thawed the tubes containing the homogenate were incubated at room temperature (15-25 °C) for 5 minutes to promote the dissociation of nucleoprotein complexes. 0.2 ml of chloroform (molecular grade; Sigma) was added per 1 ml Qiazol Lysis Reagent added originally, and the tubes

capped and shaken vigorously for 15 seconds. The tubes were then incubated at room temperature for 2-3 minutes and then centrifuged for 15 minutes at 12,000xg at 4 °C. This allowed the separation of the sample into three phases: the upper colourless aqueous phase containing the RNA, a white interphase, and a lower red organic phase. After centrifugation the upper aqueous phase was transferred carefully into a new tube without carrying through any contamination from the other phases. 0.5 ml isopropanol (molecular grade, Sigma) was added per 1 ml Qiazol Lysis Reagent added originally and the samples mixed thoroughly by vortexing and the tubes then placed at room temperature for 10 minutes and subsequently centrifuged at 12,000xg for 10 minutes at 4 °C. The RNA pellet was visible at this stage and the supernatant was carefully removed by pipetting. Then 1 ml of 75% ethanol (molecular grade, Sigma) was added per 1 ml Qiazol originally added, and the samples centrifuged at 7,500xg for 5 minutes at 4 °C. The supernatant was then removed completely and the RNA pellet briefly air dried. Once dry the pellet was redissolved in 100 µl of RNase-free water for subsequent RNA clean-up.

2.11.2 RNA Cleanup using Qiagen RNeasy Mini Kit

RNA samples extracted using the Qiazol/chloroform/isopropanol method were also cleaned-up using the Qiagen RNeasy Mini kit as per the manufacturer's protocol, to remove any contaminating phenol that could have been carried through in the extraction outlined in section 2.10.1. All centrifugation steps were performed at room temperature. 350 µl Buffer RLT was added to the 100 µl RNA samples and mixed well. 250 µl of 100% ethanol (molecular grade, Sigma) was added to the diluted RNA and mixed well by pipetting. The samples were then transferred to RNeasy Mini spin columns placed in 2 ml collection tubes. The lids were closed and the columns centrifuged for 15 seconds at 12,000xg to allow the RNA to bind to the membrane. Then the flow through was discarded and the same collection tube replaced for re-use. 500 µl Buffer RPE was added to the RNeasy spin columns, the lid closed and the columns centrifuged for 15 seconds at 12,000xg to wash the membrane. Another 500 µl of Buffer RPE was added to the spin columns and the columns centrifuged for 2 minutes at 12,000xg to ensure the membrane was dry. The RNeasy spin columns were then placed into fresh 2 ml collection tubes and

centrifuged for 1 minute at 12,000xg to avoid any possible carryover of Buffer RPE. Next the RNeasy spin columns were placed in new 1.5 ml collection tubes, and 40 µl RNase-free water added directly to the spin column membrane. The columns were then centrifuged for 1 minute at 12,000xg to elute the RNA. RNA was then transferred to fresh RNase-free tubes, the concentration measured using the Nanodrop (ThermoScientific), and then stored at -80 °C.

2.11.3 RNA extraction using the Qiagen RNeasy Mini Kit

Th1 and Th17 *in vitro* polarised cells used to investigate the mRNA expression levels of various genes by RT-qPCR were simply washed in cold PBS (~$5x10^6$ cells), centrifuged at 12,000xg for 5 minutes, the supernatant removed and the cell pellets stored at -80 °C for subsequent RNA analysis. The RNA from these samples was extracted using the RNeasy Mini Kit (Qiagen) directly as outlined here. All centrifugation steps here were performed at room temperature. The cell pellet was first thawed on ice briefly and the pellet loosened by flicking the tube. 600 µl of Buffer RLT was added to the cell pellets and mixed thoroughly by pipetting up and down 20 times. Next one volume (i.e. 600 µl) of 70% ethanol (Sigma) was added to the lysate and mixed well by pipetting. 700 µl of the samples were then transferred to the RNeasy spin columns placed in a 2 ml collection tube. The tubes were centrifuged for 15 seconds at 12,000xg and the flow-through discarded. This process was repeated, with the rest of the sample volume applied to the same columns, and centrifuged. Next 700 µl of Buffer RW1 was added to the RNeasy spin columns and the columns centrifuged for 15 seconds at 12,000xg and the flow-through discarded. 500 µl of Buffer RPE was then added to the RNeasy spin columns, the columns centrifuged for 15 seconds at 12,000xg and the flow-through discarded. Another 500 µl of Buffer RPE was added to the RNeasy spin columns and the tubes centrifuged for 2 minutes at 12,000xg to allow the membrane to dry. After this the RNeasy spin columns were transferred to fresh 2 ml collection tubes and centrifuged for 1 minute at 12,000xg to prevent any possible carryover of Buffer RPE. The RNeasy spin columns were then placed in 1.5 ml collection tubes and 40 µl RNase-free water added directly to the column membrane. The tubes were centrifuged for 1 minute at 12,000xg to elute the RNA. RNA was then transferred to fresh RNase free

tubes, concentration measured using the Nanodrop (ThermoScientific) and stored at -80 °C.

2.11.4 Reverse Transcription of RNA to cDNA for Real-Time qPCR

To perform reverse transcription of RNA to cDNA, the QuantiTect Reverse Transcription Kit (Qiagen) was used as per the manufacturer's protocol. To do this, template RNA was first thawed on ice, and the kit components, the gDNA wipeout buffer, Quantiscript Reverse Trascriptase, Quantiscript RT Buffer, RT Primer Mix and Rnase-free water thawed at room temperature (15-25 °C) and each solution mixed by flicking the tubes. First the samples were treated with a genomic DNA Wipeout buffer which eliminated any possible contaminating genomic DNA in the RNA samples. The genomic DNA elimination reactions were prepared on ice according to Table 2.2. Once the reactions were prepared, the samples were incubated for 2 minutes at 42 °C and placed immediately on ice afterwards. Next the reverse-transcription master mix was prepared on ice as a master mix according to Table 2.3 and stored on ice. 6 µl of the master mix was added to each template RNA (i.e. the whole genomic DNA elimination reaction) and each tube mixed and stored on ice. The samples were then incubated for 30 minutes at 42 °C for the reverse transcription reaction to occur, followed by 3 minutes at 95 °C to inactivate the Quantiscript Reverse Transcriptase. The cDNA samples were then stored at -20 °C for subsequent real-time PCR analysis.

2.11.5 Quantitative real-time PCR using SYBR Green

For the real-time PCR reaction, the QuantiTect SYBR Green PCR Kit was used (Qiagen) using SYBR Green I as per the manufacturer's protocol. Firstly the 2x QuantiTect SYBR Green PCR Master Mix was thawed on ice, as well as template cDNA, primers and RNase-free water, and each solution mixed thoroughly. The reaction mix was prepared for each primer set according to Table 2.4. A final concentration of 0.3 µM was used for all primers. A total reaction volume of 20 µl was utilised per reaction, using 2 µl template cDNA. The cDNA was first pipetted into the plate, and 18 µl of the master mix containing the SYBR Green PCR master

mix, primers and water was added to this. The volume of cDNA added should not exceed 10% of the final PCR volume. Any bubbles were removed from each reaction, and the plate was sealed with adhesive film and briefly centrifuged for a few seconds to ensure the contents of each well was at the bottom. The real-time cycler was programmed according to Table 2.5 and the plate containing the samples placed in the machine and the program started.

The StepOne Real-Time PCR System (Applied Biosystems) and the ABI PRISM 7000 (Applied Biosystems) were used. When using the ABI PRISM reaction volumes of 50 μl were used. Melting curve analysis was performed for each reaction to verify the specificity of the PCR primers and products. All the primers used for SYBR Green real-time PCR are listed in Table 2.6. When choosing primers, it was ensured that targets were 100-150 bp pairs in length to maximise the efficiency of the real-time PCR using SYBR Green I.

2.11.6 RT-qPCR data analysis

Data was analysed using the ΔΔCT method of relative quantification (comparative method) which measures the relative changes in gene expression (Livak and Schmittgen, 2001). All samples were normalised to the housekeeping gene hypoxanthine-guanine phosphoribosyltransferase (HPRT). The cycle threshold (CT) value for each sample denoted the cycle number at which the exponential amplification reached the threshold level (the threshold level was the chosen point at which all genes were in the exponential amplification phase). The ΔCT for each sample and the calibrator (control) sample was first calculated (ΔC_T sample = C_T target gene – C_T reference gene). Next the $\Delta\Delta C_T$ value for each sample was obtained ($\Delta\Delta C_T$ = ΔC_T sample – ΔC_T calibrator). Finally, the normalised target gene expression level in each sample was calculated as $2^{-\Delta\Delta CT}$. The $2^{-\Delta\Delta CT}$ value for the control sample is always 1.

2.12 Statistics

All statistics was performed using Prism software. When two parameters were being compared, the non-parametric Mann Whitney test was used with the following significance levels ('*' denoting $p<0.05$, ** denoting $p<0.01$, and *** denoting $p<0.001$). When three or more groups were being compared the non-parametric ANOVA, the Kruskal-Wallis (KW) test, was used to determine if there was any significance between the groups, and the Dunn's multiple comparison post test to compare the different groups. When a KW test is used and shows a significant difference between the groups, the p value for this is indicated in the figure legend, and the significance level (if any) of the Dunn's post test indicated in the figure. Due to the low numbers in the groups analysed these non-parametric tests were used, as opposed to their parametric versions. This allowed for more stringent significance testing when the normal distribution of data could not be assumed due to low sample numbers.

To determine significance in EAE scores/disease courses, a Fisher's exact test was used. This compared the disease severity between two groups (e.g. number of mice with score 3 and below, versus number of mice with score 4 and above) to determine if a proportion of mice with severe EAE was significantly different. The severity cut off point was determined for each separate experiment (due to the variability across different experiments) based on the observed severity for that experiment. The Mann Whitney test, as discussed above, was also used to determine whether there was a significant difference in the time of disease induction between two different groups.

Data shown is representative of the number of experiments indicated. However, different experiments could not be pooled together to increase dataset sizes due to the variability in EAE in terms of disease severity and/or start date.

Antibody	Conjugate	Clone	Dilution	Concentration
CD4	-AF700 (Invitrogen)	RM4-5	1:200	0.5 mg/ml
CD44	-FITC	IM7	1:200	0.5 mg/ml
CD62L	-FITC; -PE	Mel-14	1:200	0.5 mg/ml
CD25	-APC	7D4	1:200	0.5 mg/ml
CD11b	-APC; -eF450	M1/70	1:200	0.2 mg/ml
α4-integrin	-FITC (BD Pharmingen)	R1-2	1:200	0.5 mg/ml
CCR2	-PE (SantaCruz)	E68	1:200	0.5 mg/ml
CCR3	-FITC (R&DSystems)	83101	10 µl per test	25 µg/ml
CCR4	-PE (SantaCruz)	CKR-4 (H-48)	1:200	200 µg/ml
CCR5	-PE	HM-CCR5-(7A4)	1 µl per test	0.2 mg/ml
CCR6	-APC (R&DSystems)	140706	10 µl per test	10 µg/ml
CXCR3	-APC (R&DSystems)	220803	10 µl per test	10 µg/ml
PSGL-1	-PE (BD)	2PH1	1:200	0.2 mg/ml
IFN-γ	-FITC	XMG1.2	1:100	0.5 mg/ml
TNF-α	-PE	MP6-XT22	1:100	0.2 mg/ml
IL-17	-APC	eBio17B7	1:100	0.2 mg/ml
FoxP3	-APC; -PE; eF450	FJK-16s	1:100	0.2 mg/ml
T-bet	-Percp-Cy5.5; -FITC (SantaCruz Biotechnology)	eBio4B10; 4B10	1:100	0.2 mg/ml
RORγt	-PE	AFKJS-9	1:150	0.2 mg/ml
CD45.1	-FITC; -PerCP-Cy5.5	A20	1:200	0.2 mg/ml
CD90.1	-PE; -APC	OX-7; HIS-51	1:200	0.2 mg/ml
IgG1 iso control	-PE; -FITC; -APC; -PerCP-Cy55	eBRG1	1:100	0.2 mg/ml
IgG2a iso control	-PE; -FITC	eBR2a	1:100	0.2 mg/ml
Armenian hamster-Ig iso control	-PE	eBio299Arm	1 µl per test	0.2 mg/ml

Table 2.1 Flow cytometry antibodies used for flow cytometry analysis.

Component	Volume per reaction
gDNA Wipeout Buffer	2 µl
Template RNA	Variable (up to a 1 µg)
RNase-free water	Variable
Total volume	14 µl

Table 2.2 Reaction components of genomic DNA elimination reaction per tube.

Component	Volume per reaction
Quantiscript Reverse Transcriptase	1 µl
Quantiscript RT Buffer	4 µl
RT Primer Mix	1 µl
Template RNA: genomic DNA elimination reaction	14 µl
Total volume	20 µl

Table 2.3 Reverse-transcription reaction components per reaction

Component	Volume per reaction
2x QuantiTect SYBR Green PCR Master Mix	10 µl
Forward Primer	Variable
Reverse Primer	Variable
Template cDNA (10-100 ng)	Variable
RNase-free water	Variable
Total volume	20 µl

Table 2.4 Reaction set up for SYBR Green quantitative PCR

Step	Time	Temperature
PCR initial activation step:	15 minutes	95 °C
Cycling conditions:		
Denaturation	15 seconds	94 °C
Annealing	30 seconds	55 °C
Extension	30 seconds	72 °C

Table 2.5 Real-time cycler conditions.

Program was cycled for 45 cycles with an integrated melt curve analysis and data collection.

Target gene	Primer Name	Sequence
T-bet	T-bet_F	CAACAACCCCTTTGCCAAAG
	T-bet_R	TCCCCCAAGCAGTTGACAGT
RORγt	RORγt_F	CCGCTGAGAGGGCTTCAC
	RORγt_R	TGCAGGAGTAGGCCACATTACA
IL-12p35	IL-12p35_R	TACTAGAGAGACTTCTTCCACAACAAGAG
	IL-12p35_F	TCTGGTACATCTTCAAGTCCTCATAGA
IL-18	IL-18_F	TCCTTGAAGTTGACGCAAGA
	IL-18_R	TCCAGCATCAGGACAAAGAA
TGF-β	TGF-β_F	CAACCCAGGTCCTTCCTAAA
	TGF-β_R	GGAGAGCCCTGGATACCAAC
IL-6	IL-6_F	ACCAGAGGAAATTTTCAATAGGC
	IL-6_R	TGATGCACTTGCAGAAAACA
IFN-γ	IFN-γ_F	GATGCATTCATGAGTATTGCCAAGT
	IFN-γ_R	GTGGACCACTCGGATGAGCTC
IL-17	IL-17_F	CTCCAGAAGGCCCTCAGACTAC
	IL-17_R	AGCTTTCCCTCCGCATTGACACAG
TNF-α	TNF-α_F	AGGGTCTGGGCCATAGAACT
	TNF-α_R	CCACCACGCTCTTCTGTCTAC
FoxP3	FoxP3_F	CTCGTCTGAAGGCAGAGTCA
	FoxP3_R	TGGCAGAGAGGTATTGAGGG
CCR6	CCR6_F	TGTTCTGCTATCTGTTCATTATCAAGA
	CCR6_R	CACGACTCGGATGGCTCTGT
C2GnT-I	C2GnT-I_F	TCA TAA CCT GGT TCA TCA TCG C
	C2GnT-I_R	CGA TCC AGC ACT GTT TCG T
HPRT	HPRT_F	GAC CGG TCC CGT CAT GC
	HPRT_R	TCA TAA CCT GGT TCA TCA TCG C

Table 2.6 Real-Time PCR Primers for SYBR Green I

All primers were obtained from MWG Biotec.

3 Th1 cells are capable of entering the non-inflamed CNS and induce EAE, whereas Th17 cells cannot.

3.1 Introduction

Classically, EAE was considered a Th1-mediated autoimmune disease with several lines of evidence leading to this conclusion. Myelin-reactive T cell clones able to induce disease appeared to have an IFN-γ-producing Th1 phenotype (Ando et al., 1989; Kuchroo et al., 1993). T cell differentiation towards a Th1 phenotype is driven by IL-12 production from DCs (Seder et al., 1993; Macatonia et al., 1995) and the *in vivo* administration of anti-IL-12 antibody significantly reduced the severity of EAE (Leonard, Waldburger and Goldman, 1995; Segal, Dwyer and Shevach, 1998). In addition to this, IL-12-deficient mice exhibited resistance to EAE induction (Segal, Dwyer and Shevach, 1998), indicating the importance of this Th1-inducing cytokine in the initiation of autoimmune disease. This apparent role of the IL-12-Th1 lineage in EAE correlated with evidence that there was an increase in $CD4^+$ Th1 cells in the serum and CSF of MS patients (Balashov et al., 1999; Teleshova et al., 2002).

IFN-γ, the key cytokine produced during a Th1 response, leads to activation and expression of adhesion molecules on endothelial cells, thereby facilitating the entry of encephalitogenic T cells across the BBB during EAE (Dore-Duffy et al., 1996). However, despite the apparent importance of the IL-12/Th1 lineage in EAE induction, IFN-γ was surprisingly found to be protective in EAE. Treatment of mice with anti-IFN-γ antibodies led to the exacerbation of disease (Billiau et al., 1988; Lublin et al., 1993) and mice deficient in IFN-γ itself or in the IFN-γR were also susceptible to disease (Ferber et al., 1996; Willenborg et al., 1996).

The discovery of the novel cytokine IL-23 shed light on this discrepancy, highlighting that IL-12 and IL-23 share a subunit, p40 (Oppmann et al., 2000). Previous experiments that had blocked IL-12 signalling had also unknowingly been blocking IL-23. Importantly, IL-23 was also found to be key to the induction of

autoimmune disease (Cua et al., 2003). It was then possible to distinguish between IL-12 and IL-23, and IL-12-deficient ($p35^{-/-}$) mice were found to be susceptible to disease whereas IL-23-deficient ($p19^{-/-}$) mice, or double-deficient ($p40^{-/-}$) mice, were protected (Cua et al., 2003). From this the IL-23-induced Th17 lineage were then described and shown to have a important role in EAE induction (Langrish et al., 2005; Park et al., 2005) with IL-17-deficient mice exhibiting reduced severity of disease (Komiyama et al., 2006).

Since the emergence of the Th17 lineage there has been an important shift towards understanding the role of Th17 cells in disease pathogenesis with aims to developing therapeutics specifically targeting Th17 cells. At the start of this project, there was no robust method for the generation of pure populations of IFN-γ^+ Th1 cells or IL-17^+ Th17 cells devoid of any contaminating cells. Secondly, the role of IL-23 in Th17 differentiation was controversial with it being generally considered to be essential for differentiation of naïve T cells towards a Th17 phenotype (Langrish et al., 2005). It was later determined that IL-23 was required more for the stabilisation of the already-differentiated Th17 cells (Stritesky, Yeh and Kaplan, 2008; McGeachy et al., 2009).

The ability Treg to suppress IFN-γ production, but not IL-17 production *in vitro* (O'Connor, Malpass and Anderton, 2007) led to the central hypothesis that Th17-induced EAE resulted in more severe chronic EAE, due to the lack of Treg-mediated suppression. To investigate this it was important to establish disease induced by either Th1 or Th17 cells and to elucidate the contributions that each made to disease pathology. This results chapter attempts to do this.

It is not fully understood what cytokines are first upregulated in response to antigen during an immune response in the lymph node. Naïve $CD4^+$ T cells are activated when presented with antigen by the APCs within the draining lymph node. Depending on the immediate cytokine milieu in their environment, $CD4^+$ T cells differentiate into effector Th1, Th2 or Th17 cells, or into inducible Treg. As mentioned earlier, DC derived IL-12 is the main driver towards a Th1 phenotype

(Seder et al., 1993; Macatonia et al., 1995). The presence of IL-6 and IL-23 skews towards differentiation to a Th17 phenotype (Zhou et al., 2007; Stritesky, Yeh and Kaplan, 2008), whereas TGF-β is required for the differentiation into FoxP3$^+$ Treg (Zhou et al., 2008). The final aim of this chapter attempts to investigate what cytokines are upregulated in the lymph node after immunisation with peptide and CFA, and to determine whether there is a skewing towards a Th1 or a Th17 phenotype during this time.

3.1.1 Aims

The aims of this chapter were: a) to establish rigorous protocols for the *in vitro* generation of myelin-reactive Th1 and Th17 cells, b) to establish EAE driven by either Th1 or Th17 cells; c) to investigate the differences in disease pathogenesis of Th1- or Th17-driven EAE, if any were observed; d) to determine if there was a skewing towards a Th1 or Th17 phenotype after the *in vivo* activation of naïve T cells when using EAE-inducing protocols, by analysis of both innate and T cell derived cytokines.

3.1.2 Approach

Once protocols to produce pure populations of IFN-γ$^+$ Th1 cells and IL-17$^+$ Th17 cells were established, the pathogenic capability of Th1 and Th17 cells were investigated through the passive transfer model, using the polyclonal pMOG C57BL/6 model and the TCR transgenic Tg4 model. This allowed the investigation of the ability of populations of Th1 and Th17 cells to induce disease *in vivo*. The polyclonal passive transfer model involved the subcutaneous immunisation of C57BL/6 mice with pMOG and CFA. The draining lymph nodes were collected ten days later, and these primed T cells re-activated *in vitro* in the presence of pMOG and polarised towards a Th1 or Th17 phenotype. For the Tg4 passive transfer model, naïve MBP-reactive CD4$^+$ Tg4 cells were polarised *in vitro* towards a Th1 or Th17 phenotype in the presence of MBP (Ac1-9). The transferred donor cells were traceable due to the presence of congenic markers (CD45.1 or CD90.1). For analysis

spleen and CNS samples were taken at indicated time-points, and the cytokine production in response to antigen stimulation determined by flow analysis.

Two approaches were used to determine if there was a skewing towards a Th1 or Th17 phenotype after immunisation *in vivo*. Firstly, C57BL/6 mice were immunised with pMOG/CFA or with CFA alone. The lymph nodes (draining and non-draining) were sampled at various time-points to investigate the mRNA expression of a selection of innate cytokines and T cell derived cytokines, as well as the lineage specific transcription factors. The level of expression was compared to that in non-immunised naïve controls. Secondly, naïve Tg4 cells were transferred into host mice and these immunised with MBP (Ac1-9) the following day to induce EAE. Samples were then taken at day 6 post-immunisation to investigate the phenotype of the naïve T cells and determine their *in vivo* polarisation status by flow cytometry analysis.

3.2 Results

3.2.1 Optimisation of Th1 and Th17 polarising protocols of pMOG-reactive cells

The optimal protocol for polarising pure Th1 or Th17 cells *in vitro* was investigated in order to then go on and establish both Th1 and Th17 induced EAE. To do this, pMOG-reactive cells were polarised *in vitro* under various conditions (Figure 3.1 A). Not all the cells in the population produce the cytokines investigated, therefore there was always a proportion of 'cytokine negative' cells, producing neither IFN-γ or IL-17. Polarising towards a Th1 phenotype using IL-12, or IL-18, alone (with the addition of IL-2) produced a level of IFN-γ^+ cells above that of medium alone, however below the level of IFN-γ^+ cells produced after stimulation in the presence of pMOG with no exogenous cytokines. In addition to this, IL-17+ contaminants were evident, either as IL-17^+ or IL-17^+ IFN-γ^+ ('double-positive') cells after polarisation with IL-12 or IL-18 alone. However, polarising with IL-12 and IL-18 together gave rise to the highest level of IFN-γ^+ cells, above that produced in the presence of pMOG alone, and using this combination also decreased the numbers of IL-17^+

contaminating cells. Therefore, a combination of IL-12 and IL-18 was used for all future experiments to polarise towards a Th1 phenotype, (with the addition of IL-2 to promote survival). This protocol consistently generated a population with high proportions of IFN-γ^+ cells and low or negligible numbers of IL-17^+ cells.

For polarisation towards a Th17 phenotype, using IL-6 alone produced a very mixed population, with equal proportions of IL-17^+ cells and IFN-γ^+ cells and a few double producers, as did polarising with TGF-β alone, although this generated lower levels of cytokine producing cells. In contrast, using IL-23 alone gave rise to high numbers of IL-17^+ cells, but there was still a clear proportion of IFN-γ^+ cells in the form of both single and double-positive cells. Combinations of the three cytokines were trialled. Using IL-23 and IL-6 together produced the highest level of IL-17^+ cells, but again this population had IFN-γ^+ contaminating cells within it. A combination of all three polarising cytokines (IL-6, TGF-β and IL-23) gave the highest proportions of IL-17^+ cells and reduced levels of IFN-γ^+ contaminants. Therefore, for all future Th17 polarisations IL-6, IL-23 and TGF-β were used to polarise towards a Th17 phenotype.

Th1 cells polarised using IL-12 and IL-18 were T-bet^+ (Figure 3.1 B) and RORγt^- (Figure 3.1 C). Reciprocal to this, Th17 cells polarised with IL-6, IL-23 and TGF-β were T-bet^- (Figure 3.1 B) and RORγt^+ (Figure 3.1 C). The mRNA levels of T-bet and RORγt in the *in vitro* polarised cells were also determined by RT-qPCR as compared to control Th0 cells. Th1 cells had high T-bet levels (Figure 3.1 D) and low levels of RORγt expression (Figure 3.1 E). In contrast, Th17 cells, polarised using either IL-6+TGF-β+IL-23 (Th17), or using IL-6 and TGF-β only, had highly upregulated levels of RORγt (more than 30-times upregulated compared to the controls and Th1 cells) (Figure 3.1 E) and downregulated T-bet expression (Figure 3.1 D).

3.2.2 Optimisation of Th1 and Th17 *in vitro* polarised Tg4 cells

As shown in Fig. 3.1, a combination of IL-12, IL-18 and IL-2 could efficiently polarise towards a Th1 phenotype, whereas a combination of IL-23, IL-6 and TGF-β generated a Th17 phenotype. To deduce if the same was found in the TCR transgenic system, naïve Tg4 cells were FACS sorted and cultured in the presence of polarising cytokines (Figure 3.2 A). The same conditions identified using the pMOG system, gave the optimal results using the Tg4 system. There was a large variability in the level of cytokine producing cells between different experiments. As shown in Figure 3.2 A, the percentage of cytokine producing cells was lower in the MBP (Ac1-9) system compared to the pMOG system (Figure 3.2 A). However, this was not always the case. The reason for this variability is not clear, but the challenges of intracellular cytokine staining are likely to be a contributing factor. The Tg4 Th17 cell polarisation in the presence of IL-23 alone generated more IFN-γ^+ cells compared to IL-17^+ cells, although the level of cytokine production here was very low. In this case polarising Th17 cells using IL-23 and IL-6 gave rise to the highest proportion of IL-17^+ cells, but this was not consistent across different polarisation experiments. A combination of IL-6, IL-23 and TGF-β gave the most consistent results of high levels of IL-17^+ cells and very few IFN-γ^+ contaminants (Figure 3.2 A).

T-bet and RORγt mRNA levels were determined by RT-qPCR on Tg4 cells polarised under the various conditions outlined (Figure 3.2 B). T-bet was upregulated on cells polarised with IL-12 or IL-18 alone and when both were used in combination (Figure 3.2 B, left) as compared to the Th17 control. Interestingly, the Th0 cells also had upregulated T-bet expression levels. Cells cultured under Th0 conditions always had IFN-γ^+ cells in the population, so the upregulated T-bet expression was not surprising. In contrast, RORγt was upregulated on cells cultured with various Th17-associated cytokine conditions (Figure 3.2 C) compared to the Th1 control cells and it was also upregulated on the Th0 cells. Interestingly, cells 'polarised' using IL-23 alone, had upregulated levels of both T-bet and RORγt (Figure 3.2 B and C) as compared to their respective controls highlighting the heterogeneity of this cell

population. In addition, IL-23 was required to enhance RORγt expression above that in Th0 cells.

In both systems the cytokine production correlated with the expression of the appropriate transcription factor, i.e. T-bet$^+$ cells produced IFN-γ, and RORγt$^+$ cells produced IL-17 (data not shown). Comparing the two approaches, despite the pMOG-reactive cells being initially primed *in vivo*, the polarisation they received *in vitro* was still able to push the previously *in vivo* skewed cells towards the appropriate phenotype, in the same way as polarising naïve T cells. In fact, the *in vivo* primed cells appeared to exhibit a more stable phenotype as discussed later in this chapter, compared to those cells that were stimulated with their cognate antigen for the first time *in vitro*.

3.2.3 pMOG-reactive Th1 cells induce EAE, whereas their Th17 counterparts do not

To test the central hypothesis that Treg could not suppress Th17-induced EAE efficiently, Th17-induced EAE first had to be established. To do this, traceable (distinguishable from each other by flow cytometry based on CD45 and CD90 isotype expression) pMOG-reactive Th1 and Th17 cells were generated (as shown in Figure 3.3 A) and transferred in to C57BL/6 host mice, either alone or in combination. It was found that pMOG-reactive Th1 cells were able to induce disease when transferred alone, or in combination with Th17 cells (Figure 3.3 B). Surprisingly however, pMOG-reactive Th17 cells could not induce disease when transferred alone (Figure 3.3 B).

3.2.4 Disease induction correlates with the entry of donor T cells into the CNS

Donor CD45.1$^+$ Th1 cells could clearly be seen in the spleen and CNS of Th1-only transferred mice, as well as the Th1 + Th17 co-transferred mice (Figure 3.4 A and

B). Quantitatively, either population was clearly found in the spleen, when transferred alone or together (Figure 3.4 C). A significant number of donor CD90.1$^+$ Th17 cells were located in the CNS when co-transferred with the Th1 cells (Figure 3.4 D). When transferred alone, however, the Th17 cells appeared unable to enter the CNS, or at least unable to establish a population in the CNS (Figure 3.4 D).

3.2.5 Both pMOG-reactive Th1 and Th17 transferred populations did not switch phenotype *in vivo*

After an over-night *in vitro* restimulation in the presence of pMOG, intracellular cytokine staining of the transferred Th1 populations revealed high percentages of IFN-γ^+ donor cells in the CNS when the cells were transferred alone or together with Th17 cells (Figure 3.5 A and B, left and middle panels). No IL-17$^+$ cells were evident in the Th1 donor populations. Th17 cells, when able to gain access to the CNS, also appeared stable and continued to produce IL-17, but not IFN-γ, in response to pMOG stimulation (Figure 3.5 A and B, right panel). This is represented quantitatively in comparison to the spleen showing IFN-γ production by the Th1 donor cells ((25-30% IFN-γ^+ in the spleen and 15-20% IFN-γ^+ in the CNS) (Figure 3.6 A and C), with no significant numbers of IL-17$^+$ cells in the CNS or the spleen (Figure 3.6 B and D) in the Th1 donor cells. Th17 donor cells in the spleen or CNS did not switch towards a Th1 phenotype but continued to produce IL-17 and not IFN-γ (10-15% IL-17$^+$ cells in the spleen and ~8% IL-17$^+$ cells in the CNS) (Figure 3.6 B and D). Importantly, there were negligible numbers of IFN-γ^+ contaminants in the Th17 donor populations (Figure 3.6 A and C).

3.2.6 TCR transgenic Tg4 Th1 cells induce EAE, whereas Th17 cells do not

The above observations in the pMOG passive transfer model indicated surprisingly that the Th1 cells, rather than Th17 cells, were the prime initiators of EAE. To determine whether this was also the case using myelin responsive T cells receiving primary activation *in vitro*, Tg4 TCR transgenic mice were used (Figure 3.7 A and

B). Consistent with results from the pMOG passive transfer model, Tg4 Th1 cells induced disease whereas the Th17 cells did not (Figure 3.7 C). The Th1 cells induced monophasic classical EAE with the onset of disease around day 9 post-transfer (Figure 3.7 C). Samples were taken from the spleen and CNS at day 16 post-transfer. There were significantly higher total viable cell numbers (Figure 3.7 D, top) and $CD4^+$ cells (Figure 3.7 E, top) in the spleen in the Th17 transfer compared to the Th1 transfer, however the absolute number of donor cells in the spleen was comparable between the two groups (Figure 3.7 F top). An inability of the Th17 cells to enter the CNS mimicked that seen in the pMOG passive transfer model. There were lower total cell numbers in the CNS of the Th17 transfer (Figure 3.7 D, bottom), and significantly fewer $CD4^+$ T cells (Figure 3.7 E, bottom) and donor T cells in the CNS of the Th17 transfer compared to the Th1 transfer (Figure 3.7 F, bottom).

3.2.7 Tg4 Th1 cells migrate to the CNS as early as day 3 post-transfer

In order to determine how early Th1 cells can be found in the CNS in the Tg4 passive transfer model, EAE was induced using Tg4 Th1 cells and samples taken at days 3, 6 and 9 post-transfer (Figure 3.8 A, B and C). As early as day 3 post-transfer, the Th1 donor cells were approximately 10% of the $CD4^+$ population within the CNS (Figure 3.8 D, left). This percentage increased to 30% at day 6 and to over 60% by day 9 post-transfer (Figure 3.8 D, left). This increasing proportion of Th1 donor cells in the CNS correlated to increasing absolute numbers of donor Th1 cells in the CNS over time (Figure 3.8 D, right). There were roughly 200 Th1 donor cells present in the CNS at day 3 post-transfer with a 100-fold increase by day 6 (to 20,000 cells), and reaching a further 10-fold increase by day 9 to approximately 200,000 Th1 donor cells in the CNS (Figure 3.8 D, right). This total number of donor Th1 cells at day 9 post-transfer was still ~60-70% of the $CD4^+$ population in the CNS at this time-point, indicating that approximately 100,000 host $CD4^+$ T cells had also been recruited to the CNS.

3.2.8 Tg4 Th1 cells appeared phenotypically stable *in vivo*

After an over-night *in vitro* re-stimulation in the presence of MBP (Ac1-9), the cytokine production by the donor Th1 cells was determined for the CNS and spleen sampled at day 9 post-transfer. Donor Th1 cells could be clearly detected in the spleen (Figure 3.9 A) and the CNS (Figure 3.9 B). Cytokine staining revealed high percentages of IFN-γ^+ cells in the donor cells in both the spleen and the CNS (Figure 3.9 C and D). No IL-17^+ cells were apparent in either tissue (Figure 3.9 C and D) indicating the stability of the donor Th1 cells *in vivo*.

3.2.9 Deducing a dose of Tg4 Th1 cells for induction of sub-optimal EAE

Results using the pMOG model (Section 3.2.4) indicated that, once the BBB had been made more 'permeable' by Th1-induced inflammation, Th17 cells could gain access to the CNS. If so, could the Th17 cells influence disease progression or severity? To develop this, a sub-optimal number of Th1 cells were to be co-transferred with Th17 cells to allow the co-transferred Th17 cells access to the CNS. Therefore a dose of pathogenic Tg4 Th1 cells that would give rise to low level, or sub-optimal, disease had to be determined. Th1 cells were polarised and a range of Th1 cells (from $0.2x10^6$ up to $4x10^6$ blasts) were transferred (Figure 3.10 A and B). The highest number of Th1 blasts transferred ($4x10^6$) correlated with the highest severity of disease (Figure 3.10 C) and disease severity and incidence decreased with decreasing numbers of Th1 blasts transferred (Figure 3.10 C). A dose of $1x10^6$ Th1 cells gave a low level of EAE severity and this dose was used for further experiments.

3.2.10 Transfer of a sub-optimal number of Th1 cells together with Th17 cells resulted in the increase in EAE severity

Based on the above results, a sub-optimal dose ($1x10^6$) of Th1 cells was co-transferred with a high number ($4x10^6$) of Th17 cells to determine if, once the Th17 cells gained access to the CNS they were able to influence EAE severity (Figure 3.11 A). The relevant control doses of Th1 and Th17 cells were also included i.e. high ($4x10^6$) dose of Th1 cells, a low ($1x10^6$) dose of Th1 cells and a high ($4x10^6$) dose of Th17 cells. The pre-transfer phenotype of the cells was determined by intracellular cytokine staining after PMA re-stimulation for IFN-γ, IL-17 and TNF-α, and by ELISA for IFN-γ and IL-17 (Figure 3.11 B and C). The FoxP3 expression levels on the Th1 and Th17 cells were determined and found to be comparable (Figure 3.11 D).

The $Th1^{hi}$ transfer, as expected, gave a significantly increased severity of disease compared to the $Th1^{lo}$ transfer (Figure 3.12 A). The $Th17^{hi}$ transfer resulted in no induction of disease. However, when the $Th17^{hi}$ cells were co-transferred with a sub-optimal number of Th1 cells, the disease severity observed was comparable to that induced by the high number of Th1 cells alone (Figure 3.12 A) indicating the Th17 cells were able to contribute to pathology once this was initiated by Th1 cells.

3.2.11 A significant number of Th17 cells were able to enter the CNS when co-transferred with a low number of Th1 cells

The location of the donor Th1 and Th17 cells was determined from samples taken on day 20 post-transfer. In the spleen the total number of viable cells was comparable across the four groups (Figure 3.12 B), as was the percentage and absolute number of $CD4^+$ cells (Figure 3.12 C). A significantly lower percentage and absolute number of donor Th1 cells were found when a lower number were transferred compared to the Th1 high transfer (Figure 3.12 D). Comparable percentages and absolute numbers of Th17 cells were found in the spleen (Figure 3.12 E).

Within the CNS, the total number of viable cells was significantly lower in the $Th17^{hi}$ transfer, compared to the $Th1^{hi}$ transfer (Figure 3.13 A). The percentage and absolute number of $CD4^+$ cells was also significantly lower in the $Th17^{hi}$ transfer group (Figure 3.13 B). There were significantly fewer donor Th1 cells in the $Th1^{lo}$ transfer group compared to the $Th1^{hi}$ group (Figure 3.13 C). Importantly, fewer donor Th17 cells were found in the CNS of the $Th17^{hi}$ transfer group compared to in the $Th1^{lo}$ + $Th17^{hi}$ co-transfer group (Figure 3.13 D, right) as these cells were unable to gain access to the CNS.

In summary, there was no significant difference in the number of Th17 cells between the two Th17 groups (Figure 3.13 E) in the spleen. However, there were a significant number of Th17 cells in the CNS in the co-transferred group, compared to when the Th17 cells were transferred alone (Figure 3.13 F). This suggested the increase in the number of Th17 cells in the CNS on co-transfer was specific to the target organ. The small but significant population of Th17 cells in the CNS of the co-transferred group appeared able to increase the severity of disease observed, however the mechanism for this increase in disease severity is as yet unknown.

3.2.12 Cytokine production in the CNS during active EAE

The data shown above suggested a superior role of IFN-γ-producing Th1 cells in the induction of disease by passive transfer. During active EAE naïve T cells are activated and differentiated *in vivo* and subsequently migrate to the CNS to induce disease. To determine the dominant pro-inflammatory response (Th1 or Th17) in active EAE, mice were immunised to develop pMOG-induced active EAE (Figure 3.14 A). In the CNS at the peak of disease, the $CD4^+$ population had a mixed phenotype of IFN-γ^+, IL-17^+ and TNF-α^+ cells in response to pMOG re-stimulation (Figure 3.14 B) indicating that both Th1 and Th17 cells were at least present within the CNS at this time.

3.2.13 Both a Th1 and Th17 inflammatory response is induced after immunisation with pMOG/CFA

To determine what response is dominant after immunisation with CFA and/or pMOG/CFA, C57BL/6 mice were immunised and the level of mRNA expression above that of non-immunised controls was determined for various Th1- and Th17-associated cytokines (Figure 3.15 A). IL-12 (Figure 3.15 B), IL-18 (Figure 3.15 C), IFN-γ (Figure 3.16 A) and T-bet (Figure 3.16 B) showed upregulated mRNA expression above the non-immunised controls indicative of a Th1 response. The level of mRNA expression of IL-17 (Figure 3.16 C), TGF-β (Figure 3.15 D) and RORγt (Figure 3.16 D) were also upregulated above the non-immunised control samples indicative of a Th17 response. IL-6 mRNA was found at very low levels of expression in the immunised mice, never above the control samples (Figure 3.15 E). Interestingly FoxP3 mRNA was slightly upregulated above the controls (Figure 3.16 F). IFN-γ expression appeared early in the response (day 3) whereas IL-17 expression came up later (around day 5 and 7 post-immunisation) (Figure 3.16 A and C), suggesting that a Th1 response was induced first, followed closely by a Th17 response.

3.2.14 *In vivo* activation of naïve Tg4 T cells in response to MBP (Ac1-9) immunisation

The above results suggested that both a Th1 and a Th17 response occurred after immunisation with pMOG and CFA. To determine if this was the same in the Tg4 TCR transgenic model, naïve Tg4 cells were transferred into host mice and these were subsequently immunised with MBP (Ac1-9)/CFA to induce disease (Figure 3.17 A and B). Samples were taken on day 6 post-immunisation to determine the phenotype of the donor cells after *in vivo* activation. The $CD4^+$ population and $CD45.1^+$ donor cell population could clearly be found in the CNS, spleen and draining (inguinal) lymph nodes (Figure 3.17 C-F). Intracellular cytokine staining of the donor T cells revealed a mixed population of IFN-γ^+, IL-17^+ and TNF-α^+ cells in all tissues sampled (Figure 3.18 A) indicating differentiation of the naïve T cells towards both a Th1 and a Th17 phenotype. The proportion of $FoxP3^+$ cells was low

on the donor T cells in the CNS and spleen compared to the host population (Figure 3.18 B) suggesting the transferred Tg4 cells had not differentiated towards a regulatory phenotype. Lastly, T-bet upregulation could not be detected on the donor cells in any tissue, whereas the RORγt expression appeared upregulated on the donor cells above the isotype control, but was comparable across the tissues sampled (Figure 3.18 C). These results were indicative of polarisation of the naïve T cells towards both Th1 and Th17 phenotypes.

3.3 Discussion

It is well established that IL-12 is required for Th1 cell development (Gately et al., 1998) leading to the upregulation of IFN-γ and subsequently, T-bet, the key transcription factor of the Th1 lineage (Szabo et al., 2000; Lighvani et al., 2001). IL-12 induces the expression of IL-12Rβ2 which allows the cells to become responsive to IL-12 signalling (Langrish et al., 2005). Here, polarisation with IL-12 led to an increase in IFN-γ^+ cells. However, the addition of IL-18 to the cytokine mix increased the level of IFN-γ production by the Th1 cells and also decreased the frequency of IL-17^+ contaminants in the Th1 population. IL-18 (previously known as IFN-γ-inducing factor) has the ability to induce IFN-γ, TNF-α and GM-CSF production by T cells as well as the upregulation of IL-2Rα (Kohno and Kurimoto, 1998; Okamura et al., 1998). Therefore, for this project, the polarisation of naïve T cells, or pMOG-reactive cells towards a Th1 phenotype was routinely performed using a combination of IL-12 and IL-18. This consistently produced a T-bet^+ population (as determined at both the protein level by flow cytometry analysis, and the mRNA level by RT-qPCR) with a high proportion of IFN-γ^+ cells and, importantly, very few IL-17^+ contaminants. The use of IL-18 in the Th1 polarising cocktail increased the number of IFN-γ^+ and TNF-α^+ cells and also resulted in a more severe form of EAE being induced on transfer (Ito et al., 2003) (also see Chapter 5).

The cytokines required for the development of Th17 cells have been a topic of much debate since the description of this Th subset. Initially, it was thought that IL-23 was the key cytokine required for differentiation towards a Th17 phenotype (Cua et al.,

2003; Langrish et al., 2005) due to the high numbers of IL-17$^+$ cells induced after expansion with IL-23 alone. As shown here, expansion with IL-23 produced a population with high numbers of IL-17$^+$ cells (~30%), but also with a high number of IFN-γ$^+$ cells (~20%, both IFN-γ$^+$ single positive cells and IFN-γ$^+$ IL-17$^+$ double positive cells). Therefore, disease conferred after the transfer of IL-23 conditioned 'Th17' cells, as has been done in numerous studies (Langrish et al., 2005; McGeachy et al., 2007) would not give conclusive results as to which cell type, IL-17$^+$ Th17 or IFN-γ$^+$ Th1, had contributed to the disease induction, due to the mixed population used on input.

IL-23 is not required for the initial differentiation of Th17 cells from naïve T cells. However, it is important for the maintenance of their stability and for their terminal differentiation (Stritesky, Yeh and Kaplan, 2008; McGeachy et al., 2009). The key cytokines for the differentiation of naïve T cells towards a Th17 phenotype have been determined to be IL-6 and TGF-β (Serada et al., 2008). IL-6 has been shown to be directly needed for the differentiation into Th17 cells (Bettelli et al., 2006) and the induction of RORγt expression (Ivanov et al., 2006). TGF-β has been shown to indirectly mediate Th17 differentiation by blocking the expression of STAT4 and GATA-3, thereby preventing Th1 or Th2 differentiation (Das et al., 2009) and promoting Th17 differentiation. For this project, a combination of IL-6, IL-23 and TGF-β were used for the *in vitro* polarisation of Th17 cells as this generated the 'cleanest' cell populations with minimal IFN-γ$^+$ contaminating cells in our models.

It has been suggested that polarising Th17 cells with only IL-6 and TGF-β (i.e. in the absence of IL-23) results in the increase in IL-17, but also in the anti-inflammatory cytokine, IL-10 (McGeachy et al., 2007). Although these cells polarised in this way upregulate IL-17, they cannot induce inflammatory disease due to their production of IL-10 (McGeachy et al., 2007). Other protocols for the differentiation of Th17 cells involve an initial stimulation in the presence of IL-6 and TGF-β, followed by a rest period in the presence of IL-23, and finally a re-stimulation with anti-CD3/anti-CD28 (Jager et al., 2009) producing a Th17 cell population with high numbers of IL-17$^+$ cells. However the *in vivo* stability (cytokine phenotype) of these Th17 cells

is questionable. It has also been shown that Th17 differentiation is modulated by the activation of the aryl hydrocarbon receptor (AhR) (Veldhoen et al., 2008a). Interestingly, the culture medium Iscove's modified Dulbecco's medium (IMDM), is rich in aromatic amino acids which give rise to AhR agonists and therefore supports increased differentiation towards the Th17 phenotype. In contrast, RPMI medium, which was used here, is thought not to support high Th17 differentiation due to a lack of AhR agonists (Veldhoen et al., 2008a; Veldhoen et al., 2009). Th17 differentiation in IMDM was trialled during this project, but did not show any increased differentiation towards the Th17 phenotype in our hands, when compared to polarisation in RPMI (data not shown). In addition, numerous laboratories showing strong Th17 polarisation as well as pathogenicity have performed their *in vitro* Th17 polarisations in RPMI medium (McGeachy et al., 2007; Jager et al., 2009), showing that although AhR ligation may indeed be beneficial for Th17 differentiation, it is not essential for their differentiation and pathogenicity.

IL-1 signalling has also been suggested to be important for *in vivo* Th17 differentiation, especially for regulating the expression of IRF4 and RORγt (Chung et al., 2009). The addition of IL-1β to the Th17 polarisation cytokine mix was trialled here and, in terms of RORγt expression, it did not have a beneficial effect over the use of only IL-6, IL-23 and TGF-β, although it did further downregulate the expression of T-bet mRNA. IL-1 signalling is also required in EAE, as demonstrated by the significantly lower disease observed in IL-1R1KO mice compared to wild-type mice, due to a lack of a Th17 response (Sutton et al., 2006). In addition, IL-1β and IL-23 activated γδ T cells appear to promote IL-17 production by $CD4^+$ T cells (Sutton et al., 2009).

Once the protocol for the most pronounced polarisation of stable Th1 and Th17 cells had been established, the central hypothesis of this project could be tested. However this required the establishment of Th17-driven EAE, but Th17 cells could not induce disease when transferred alone. This was observed for pMOG-reactive Th17 cells polarised with IL-6, TGF-β plus IL-23, as well as for those polarised with just IL-6 and TGF-β (O'Connor et al., 2008).

This inability of the Th17 cells to induce disease correlated directly with their apparent inability to home to the non-inflamed CNS as opposed to their Th1 counterparts. Once inflammation had been established by the Th1 cells, the Th17 cells could then follow into the CNS, and presumably had a role to play in pathology. This inability of Th17 cells to home to the site of inflammation has also recently been demonstrated in a model of RA (Janke et al., 2010). As mentioned earlier, IL-23 is required for the terminal differentiation of Th17 cells. Numerous early studies in the role of Th17 cells in autoimmunity used IL-23 conditioned 'Th17' cells (Langrish et al., 2005; Park et al., 2005). However as shown here, polarisation with IL-23 alone resulted in a very mixed population of IL-17^+ and IFN-γ^+ cells, resulting in inconclusive data in which the key pathogenic cell type is unknown. The IL-23R is not expressed on naïve T cells and its expression is induced in the presence of IL-23 (Langrish et al., 2005). *In vivo* neutralisation of IL-23R signalling on day 2 and day 4 post-immunisation led to the impairment of *in vivo* Th17 differentiation (McGeachy et al., 2009). Neutralisation at day 6 or later had no effect on Th17 differentiation indicating that IL-23R expression and signalling was required early on in the differentiation of Th17 cells *in vivo* (McGeachy et al., 2009). Various different Th17 protocols were trialled in this project including resting the Th17 cells in IL-23 alone, after their initial differentiation with IL-6 and TGF-β. However this did not result in an enhanced Th17 differentiation in terms of increased IL-17 production and decreased IFN-γ production (data not shown). Neither did it have an effect on their ability to induce disease (data not shown).

The pMOG-reactive Th1 and Th17 cells appeared to have stable cytokine profiles *in vivo*. Transferred Th1 cells located in both the spleen and the CNS, continued to produce IFN-γ in response to pMOG stimulation, and they exhibited no capacity to produce IL-17. Reciprocally, the pMOG reactive Th17 cells continued to produce IL-17 and did not switch towards an IFN-γ-producing phenotype.

In marked contrast to this, in the Tg4 TCR transgenic system, transferred Th17 cells cultured with the same *in vitro* cytokine cocktail as the pMOG-reactive Th17 cells, but from a sorted naïve population, have been shown to occasionally be able to

induce EAE, albeit with a low severity, low incidence and delayed induction (O'Connor et al., 2008). Interestingly, whenever this observation of disease with Th17 cells was made, it clearly correlated with instability of these TCR transgenic Th17 cells, reflected in their switch towards IFN-γ production. This switch towards a IFN-γ-producing-Th1 phenotype occurred only in the CNS, presumably due to the stimulation with myelin antigen, whereas this did not occur within the spleen, where the transferred Th17 cells remained stable (O'Connor et al., 2008). This *in vivo* instability was seen when Th17 cells were transferred into both normal B10.PL mice and lymphopenic $RAG2^{-/-}$ mice (O'Connor et al., 2008). This correlates with *in vitro* observations of Th17 cells switching to IFN-γ-producing phenotype after re-stimulation with higher concentrations of antigen (O'Connor et al., 2008).

The obvious difference between the pMOG/C57BL/6 and the MBP(Ac1-9)/Tg4 passive transfer models is the nature of the initial antigen challenge i.e. *in vivo* with CFA immunisation for the C57BL/6 system, as opposed to *in vitro* for the Tg4 TCR transgenic system. The stability of the Th17 cells in the pMOG-passive transfer model suggests that the pMOG-reactive cells are exposed to an unknown cytokine or other stimulus *in vivo* during the initial priming and this confers their later stability.

Th17 cells switching towards a Th1-phenotype have been observed in both EAE and other models and this adds weight to the numerous recent publications showing Th subsets are not as stable, or static, as previously thought. In the NOD mouse model of type 1 diabetes, it was found that Th17 polarised cells were readily able to induce diabetes in NOD/SCID recipients (Bending et al., 2009). However, these Th17 cells were found to have converted to an IFN-γ-producing phenotype within the recipient mice highlighting the plasticity of this Th subset. Polarising Th17 cells using the method outlined from Vijay Kuchroo's lab (Jager et al., 2009) produces cells with a high number of IL-17^{+} cells in the population. On transfer of these cells, they were found to induce severe EAE. However, in our hands these 'Th17' cells, that show negligible numbers of IFN-γ^{+} cells on input, have a high proportion of IFN-γ^{+} cells in the population in the CNS, and reduced levels of IL-17^{+} cells again indicating a switch towards a Th1 phenotype (Richard O'Connor, personal communication). This

leads to uncertainties as to whether the disease observed is indeed due to the IL-17$^+$ Th17 cells, or due to the switch in phenotype towards a Th1 IFN-γ-secreting phenotype. Treatment of NOD/SCID mice that had Th17-induced diabetes with an IL-17 neutralizing antibody had no effect on disease, whereas treatment with a neutralising IFN-γ antibody led to the abrogation of Th1-induced disease, indicating that at least in that model, IFN-γ, or a Th1-phenotype, is more important for the induction of diabetes (Bending et al., 2009).

A simple explanation for the lack of disease observed after Th17 passive transfer would be the presence of high numbers of Treg within the transferred population. However, the transferred cells were checked for their level of FoxP3$^+$ cells, and this was found to be low in both the Th1 and Th17 polarised cells (data shown here; (O'Connor et al., 2008). Secondly, it has been shown that Th17 cells polarised with IL-6 and TGF-β alone, have higher levels of IL-10 production and these cells do not have any pathogenic potential (McGeachy et al., 2007), and even have a suppressive effect if co-transferred with a pathogenic population (McGeachy et al., 2007). The level of IL-10 in the Th1 and Th17 cultures here was verified by ELISA and cytokine bead array, and found to be very low in the Th17 cultures, and even slightly higher in the Th1 cultures (O'Connor et al., 2008). The presence of increased IL-10 could not therefore explain the lack of pathogenic potential of these Th17 cells.

As shown in the TCR transgenic Tg4 model, Th1 cells induce disease in a dose dependent manner and can be located in the CNS as early as day 3 post-transfer, well before any clinical signs of disease. The presence of the donor Th17 cells in the CNS, only when co-transferred with Th1 donor cells, suggests a model in which it is the Th1 cells that are able to cross the non-inflamed BBB into the CNS. Once inflammation is established, BBB 'permeabilisation' allows the Th17 cells and other pro-inflammatory cells to gain access to the CNS. In order to determine whether Th17 cells can have a role in EAE pathology in this TCR transgenic model, different numbers of donor T cells were transferred. When the low dose of Th1 cells and high dose of Th17 cells were transferred together, the disease observed was more severe than that of the Th1 low dose alone. Importantly, the high dose of Th17 cells, when

transferred alone, was not able to induce disease indicating that the presence of the Th1 cells was necessary, to allow the Th17 cells entry into the CNS. The Th17 donor cells clearly could not enter the CNS across the non-inflamed CNS when transferred alone. However, when transferred together with the donor Th1 cells, a small but significant population of Th17 cells was then found within the CNS. This indicates the sub-optimal number of donor Th1 cells were able to cross the BBB, to establish sub-optimal CNS inflammation, allowing the Th17 donor cells to gain access to the CNS and increase the severity of disease.

The mechanism of this disease exacerbation is still unknown with several different possibilities: 1) The donor Th17 cells could indeed be exacerbating disease directly themselves, through the production of IL-17 or another pro-inflammatory cytokine, however, it has been suggested that IL-17 is not required for disease induction (Haak et al., 2009), which would suggest the disease exacerbating agent might be another pro-inflammatory cytokine that the Th17 cells can produce; 2) the donor Th17 cells, once in the CNS, could be facilitating the Th1 cells to exacerbate disease; 3) the Th17 cells could switch towards a more Th1-phenotype in the CNS and this could be contribute to the increased severity of disease.

On immunisation to induce EAE, the peptide is presented by APCs in the draining lymph node to naïve T cells. These naïve T cells are then activated and depending on the cytokine milieu, they proliferate and differentiate towards a particular phenotype, Th1 or Th17. Subsequently these cells migrate towards the CNS where they are able to elicit an inflammatory response. This involves the production of IFN-γ, IL-17 or TNF-α, depending on their phenotype although the precise role for these cytokines remains controversial. It is not fully understood what cytokines are first upregulated within the draining lymph node after immunisation and how these may affect the drive towards a Th1 or Th17 inflammatory response. Here the mRNA expression levels of innate cytokines, and those produced directly by the T cells were investigated after immunisation with pMOG mixed together with CFA, and CFA-only controls, compared to non-immunised control mice. No difference was found between mice immunised with pMOG/CFA and CFA-only, indicating that high

amounts of cytokines were not produced by the T cells in response to pMOG itself. However, it appears the CFA promoted both the Th1 and Th17 responses. In most cases cytokines were upregulated in both the draining lymph nodes and the non-draining lymph nodes, suggesting drainage from the inguinal lymph nodes through to the axillary and brachial lymph nodes, and a systemic effect of the immunisation (Harrell, Iritani and Ruddell, 2008). IL-12 and IL-18, both Th1 inducing cytokines, were upregulated above the non-immunised controls. This was reflected by the up-regulation of IFN-γ and T-bet suggesting the presence of a Th1 response.

There was a strong upregulation of IL-17 mRNA and a slight upregulation of RORγt indicative of a Th17 response. No clear IL-6 signal was detected for some of the samples, indicating very low expression levels of mRNA. Furthermore, and notably, no clear signal at all was found for IL-23 (data not shown). There was a trend towards an upregulation ($2^{-\Delta\Delta CT}<2$) of TGF-β and FoxP3 indicating a possible increase in $Foxp3^+$ Treg after immunisation.

To determine what phenotype naïve T cells adopt after immunisation in the MBP (Ac1-9) model, naïve Tg4 cells were transferred in host mice and the mice immunised to induce EAE. Interestingly, by day 6, the donor cells had already adopted a strong inflammatory response and there were high proportions of IFN-γ^+, IL-17^+ and TNF-α^+ T cells within the donor cell population in the tissues sampled with no particular skew towards a Th1 or Th17 response. Although this time-point was pre-clinical, it would be important to look even earlier in the disease course to determine if the naïve T cells have a particular skewing towards a Th1 or Th17 phenotype when they are first activated. The data presented here would suggest that there is no skew and that equal proportions of Th1 and Th17 cells are differentiated after activation.

3.4 Conclusions

- Protocols for the polarisation of populations of IFN-γ^+ Th1 cells (using IL-12, IL-18 and IL-2), and IL-17^+ Th17 cells (using IL-6, IL-23 and TGF-β), with negligible numbers of contaminating cells in either population were established.
- The Th1 cells were clearly able to home to the CNS, crossing the non-inflamed BBB to induce EAE. In contrast the Th17 cells were unable to enter the non-inflamed CNS, and therefore did not induce disease when transferred alone.
- There was no increased numbers of FoxP3^+ Treg, or IL-10 producing Tr1 cells in the Th17 population that would account for their lack of pathogenicity.
- In the pMOG passive transfer model, when Th17 cells did gain access to the CNS (when co-transferred with Th1 cells), the Th17 cells remained stable and continued to produce IL-17 *in vivo* in both the CNS and the spleen.
- In contrast, in the TCR transgenic model, when Th17 cells gained access to the CNS, the Th17 cells appeared to be more plastic and had a high proportion of IFN-γ^+ cells in the population, within the CNS, but interestingly, not in the spleen. Therefore, *in vitro* generated Th17 cells are not stable in their phenotype, and have the ability to switch towards a more Th1-IFN-γ-producing phenotype, in comparison to Th17 cells initially primed *in vivo*.
- After *in vivo* immunisation, Th1 and Th17-associated innate and T cell-produced cytokines are upregulated in the draining and non-draining lymph nodes, indicating the initiation of both Th1 and Th17 responses.

The following two chapters explore the pathogenicity of these two T helper subsets using ‘Th1’ cells as a pathogenic subset, and ‘Th17’ cells as a non-pathogenic subset to determine how the pathogenic cells induce disease and why the non-pathogenic cells do not. Chapter 4 addresses the differential expression of homing molecules on the pathogenic and non-pathogenic cells, which could define the ‘pioneer’ cells that

first enter the CNS. Chapter 5 addresses the cytokines produced by the pathogenic (Th1) cells and the requirement for these cytokines in the clinical expression of EAE.

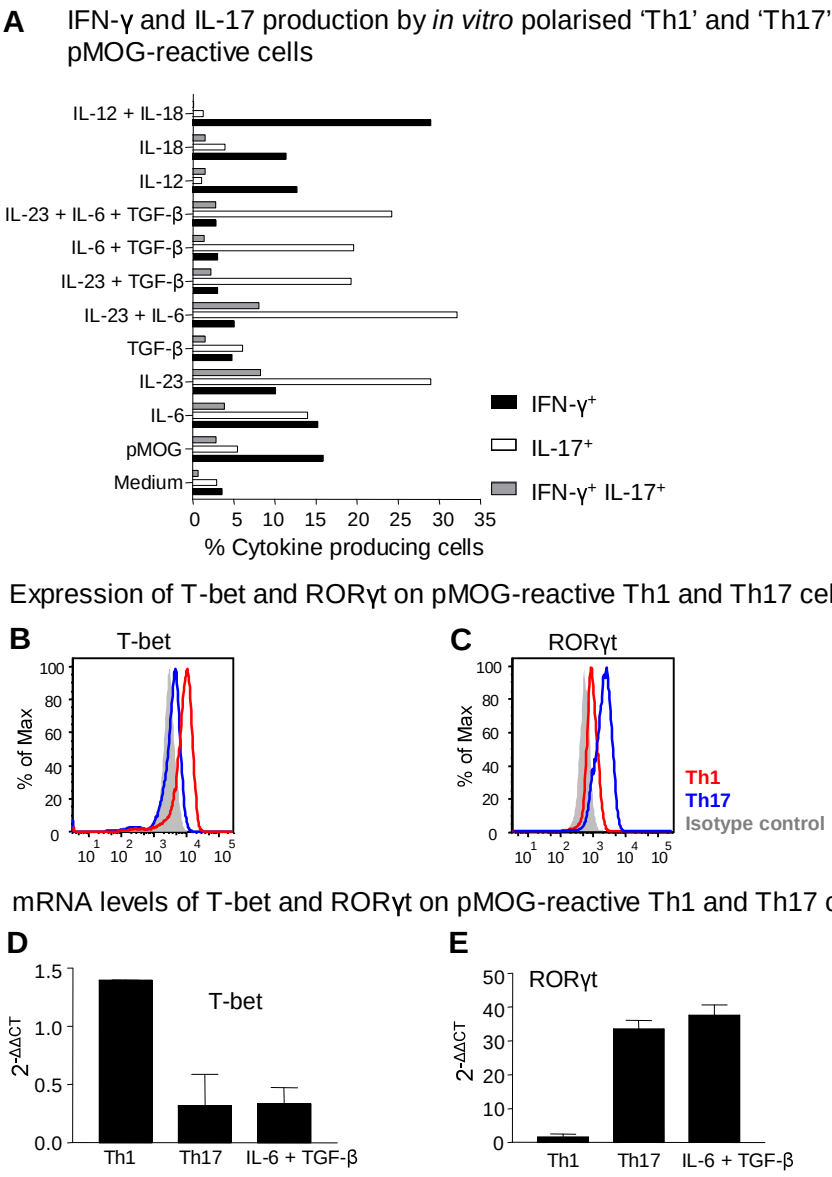

Figure 3.1 Optimisation of *in vitro* polarisation of pMOG-reactive Th1 and Th17 cells.

C57BL/6 mice were immunised with 100 µg pMOG mixed with CFA, ten days later the draining lymph nodes were harvested and the cells cultured for 72 hours *in vitro* under the conditions outlined. **A,** gated on $CD4^+$ cells, percentage IFN-γ^+ and IL-17^+ cells in pMOG-reactive cells polarised in the presence of 10 µg/ml pMOG and the indicated polarising cytokines; gated on $CD4^+$ cells, expression of **B,** T-bet and **C,** RORγt on pMOG-reactive Th1 (polarised using IL-12, IL-18 and IL-2) and Th17 (polarised using IL-6, IL-23 and TGF-β) cells showing expression on Th1 (**red**), Th17 (**blue**) cells and the isotype control (**grey fill**); mRNA expression levels of **D,** T-bet and **E,** RORγt on *in vitro* polarised Th1 cells and Th17 cells (polarised with IL-6, IL-23 and TGF-β, or with IL-6 and TGF-β only), as compared to Th0 cells (pMOG-reactive cells cultured with pMOG and no exogenous cytokines) (Th0, $2^{-\Delta\Delta CT} = 1$). Error bars represent standard error of the mean of replicate samples.

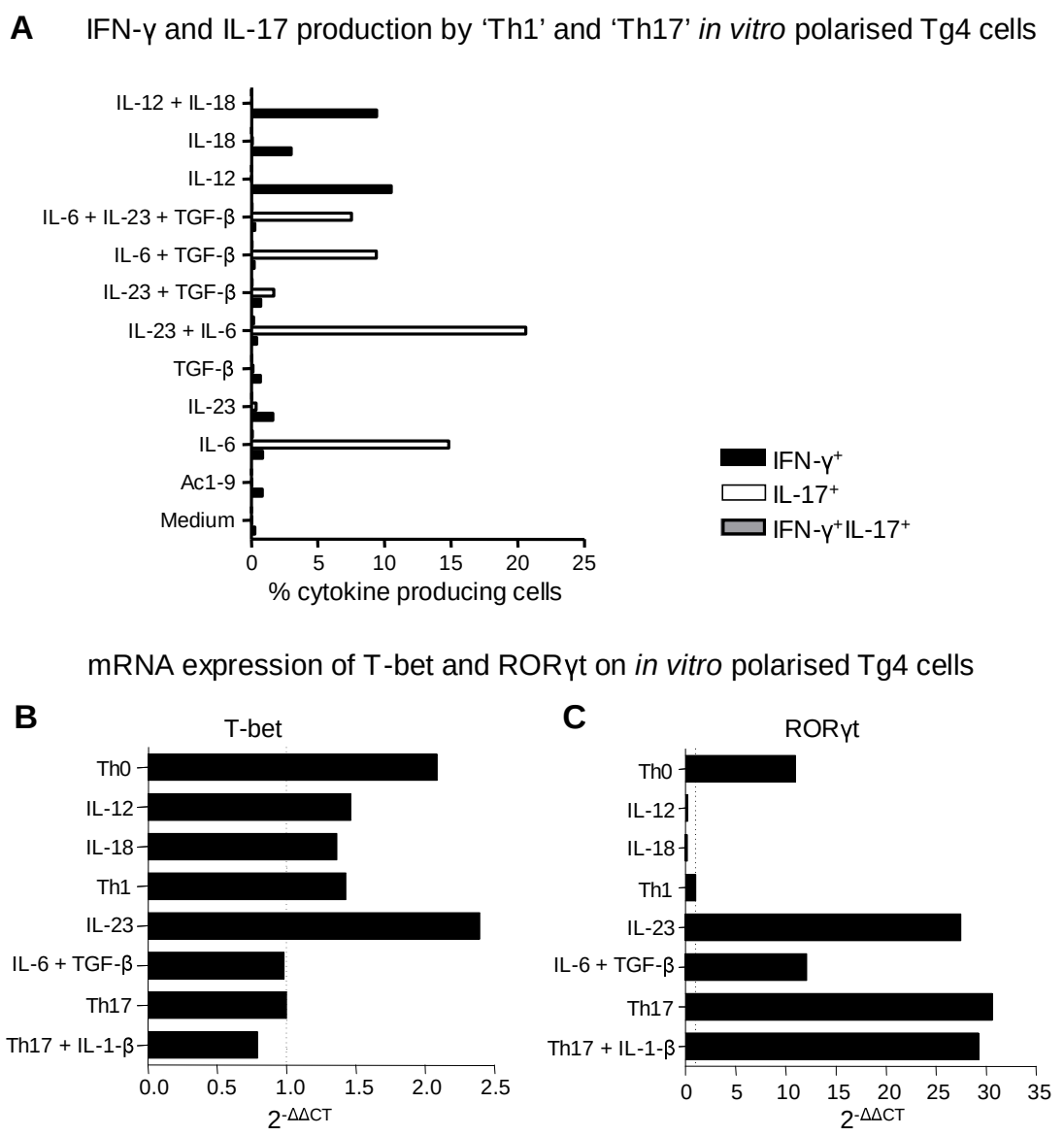

Figure 3.2 Optimisation of *in vitro* polarisation of naïve Tg4 T cells to a Th1 and Th17 phenotype.

Naïve $CD4^+$ $CD62L^{hi}$ $CD25^-$ Tg4 T cells were sorted and polarised *in vitro* in the presence of irradiated B10.PL splenocytes, 10 μg/ml MBP (Ac1-9) and the indicated polarising cytokines for 72 hours. **A,** gated on $CD4^+$ cells, percentage IFN-γ^+, IL-17^+ and IFN-γ^+ IL-17^+ cells after 72 hours polarisation under stated conditions; mRNA expression levels of **B,** T-bet and **C,** RORγt on Tg4 Th1 and Th17 cells polarised under the stated conditions. 'Th1' denotes polarisation with IL-12, IL-18 and IL-2. 'Th17' denotes polarisation with IL-6, IL-23 and TGF-β. 'Th0' denotes cells cultured in the presence of MBP (Ac1-9) with no exogenous cytokines. The T-bet control sample was Th17 polarised cells; the RORγt control sample was Th1 polarised cells. The dotted line represents the $2^{-\Delta\Delta CT} = 1$ value of the relevant control sample. Representative of two similar experiments.

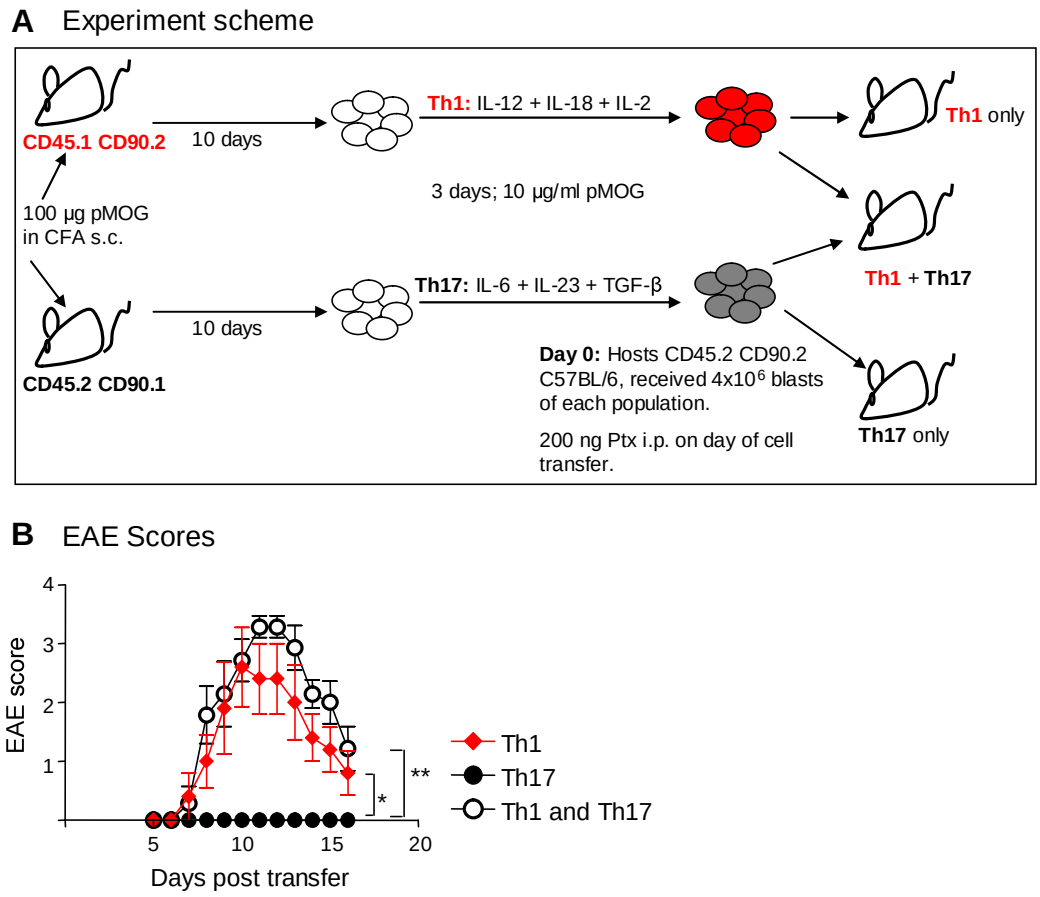

Figure 3.3 *In vitro* polarised pMOG-reactive Th1 cells induce EAE whereas Th17 cells do not.

A, Experiment scheme; **B,** Mean clinical EAE scores after transfer of Th1 only (♦), Th17 only (●), and Th1 plus Th17 (○) cells. Disease incidence: Th1 (4/5), Th17 (0/5), Th1+Th17 co-transfer (7/7). Error bars represent mean ± standard error. The proportion of mice with severe EAE is higher in the Th1 transfer versus the Th17 transfer (* p=0.0476), and in the Th1+Th17 co-transfer versus Th17 transfer (** p=0.0047). There was no significant difference in severity between the Th1 and the Th1+Th17 transfer groups (p=1.0000). Statistics performed using the Fisher's exact test comparing mice with maximum scores of 2 and below, and, 3 and above. (*In vivo* work performed by Dr Richard O'Connor due to not having a Home Office license at this stage). Representative of numerous pMOG-reactive Th1 versus Th17 passive transfer experiments.

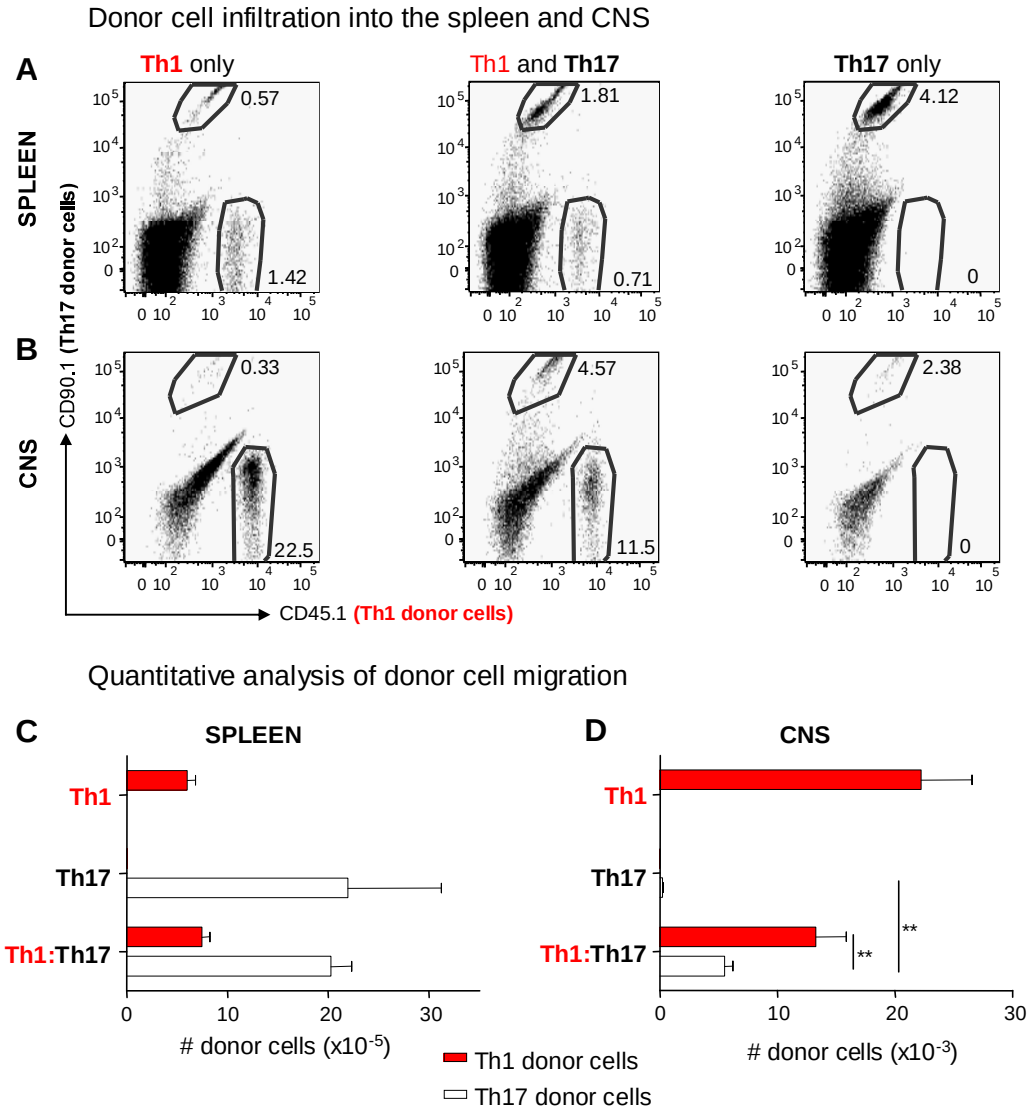

Figure 3.4 Disease induction correlates with the ability of the pMOG-reactive Th1 cells to home to the CNS, whereas Th17 cells do not do so.

Gated on the $CD4^+$ $CD11b^-$ cells, presence of the $CD45.1^+$ Th1 donor cells and the $CD90.1^+$ Th17 donor cells, in the **A,** spleen and **B,** CNS in the Th1 only transfer (left panel), Th1+Th17 co-transfer (middle panel) and Th17 only transfer (right panel) at day 16 post-transfer; Quantitative analysis showing absolute number of Th1 donor cells (**red bar**) and Th17 donor cells (**clear bar**) in the **C,** spleen and **D,** CNS in the Th1-only (Th1) and Th17-only (**Th17**) transfer, and the co-transfer (Th1:**Th17**). There was a significant difference between the number of Th1 and Th17 donor cells in the co-transfer in the CNS (** p=0.0025). There were significantly more Th17 cells in the CNS in the co-transfer compared to when transferred alone (** p=0.0061). Statistical analyses performed using Mann Whitney test ('*' represents: *p<0.05; **p<0.01; ***p<0.001). Error bars represent mean ± standard error. See Appendix 1 for flow cytometry gating strategy for donor T cells. '#' denotes 'number'. n=5 (Th1); 5 (Th17); 7 (Th1+Th17 co-transfer).

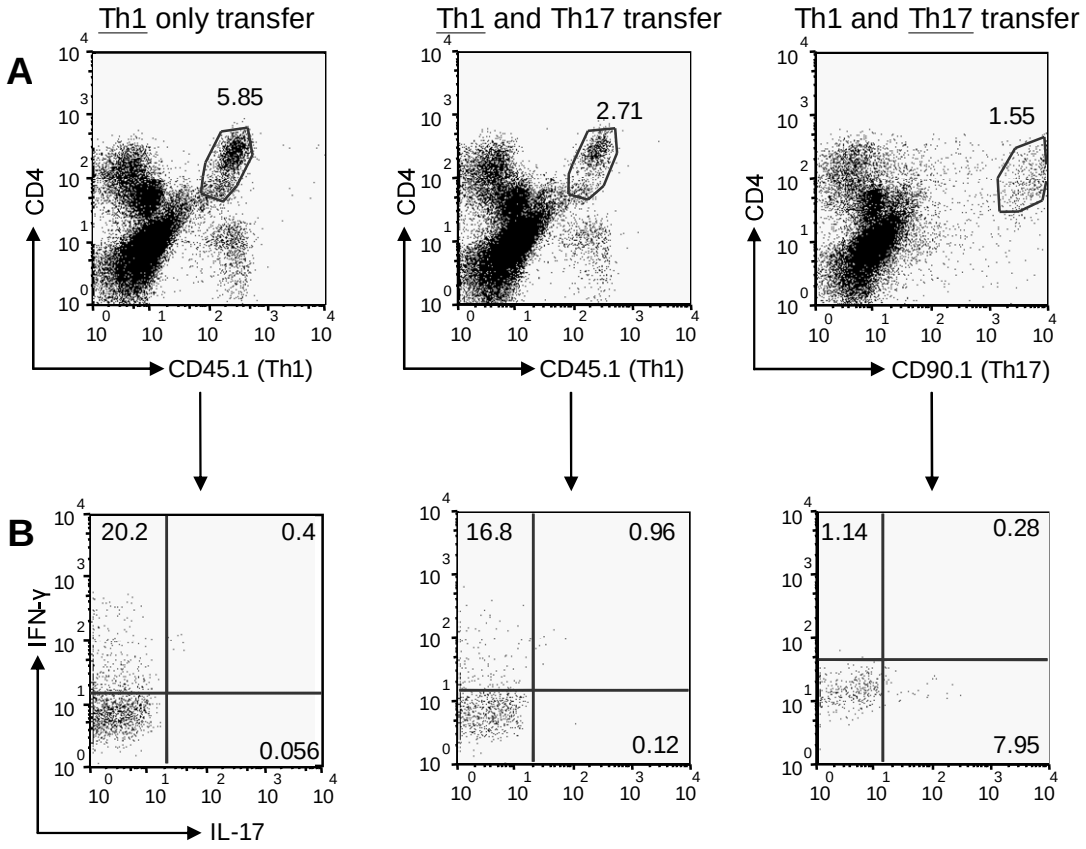

Figure 3.5 pMOG reactive Th1 and Th17 cells do not switch phenotype *in vivo*.

Production of IFN-γ and IL-17 by donor T cells after an over-night re-stimulation with 20 μg/ml pMOG, **A,** gated on $CD11b^-$ cells presence of $CD4^+$ $CD45.1^+$ Th1 donor cells in the Th1 only transfer (left panel), and in the Th1+Th17 co-transfer (middle panel), and the presence of the $CD4^+$ $CD90.1^+$ Th17 donor cells in the Th1+Th17 co-transfer (right panel); **B,** gated on each donor cell population, production of IFN-γ and IL-17 by the donor cells in each respective transfer. Underlined population represents the population being viewed. CNS samples pooled for each group and re-stimulated over-night. Gating determined on relevant isotype controls.

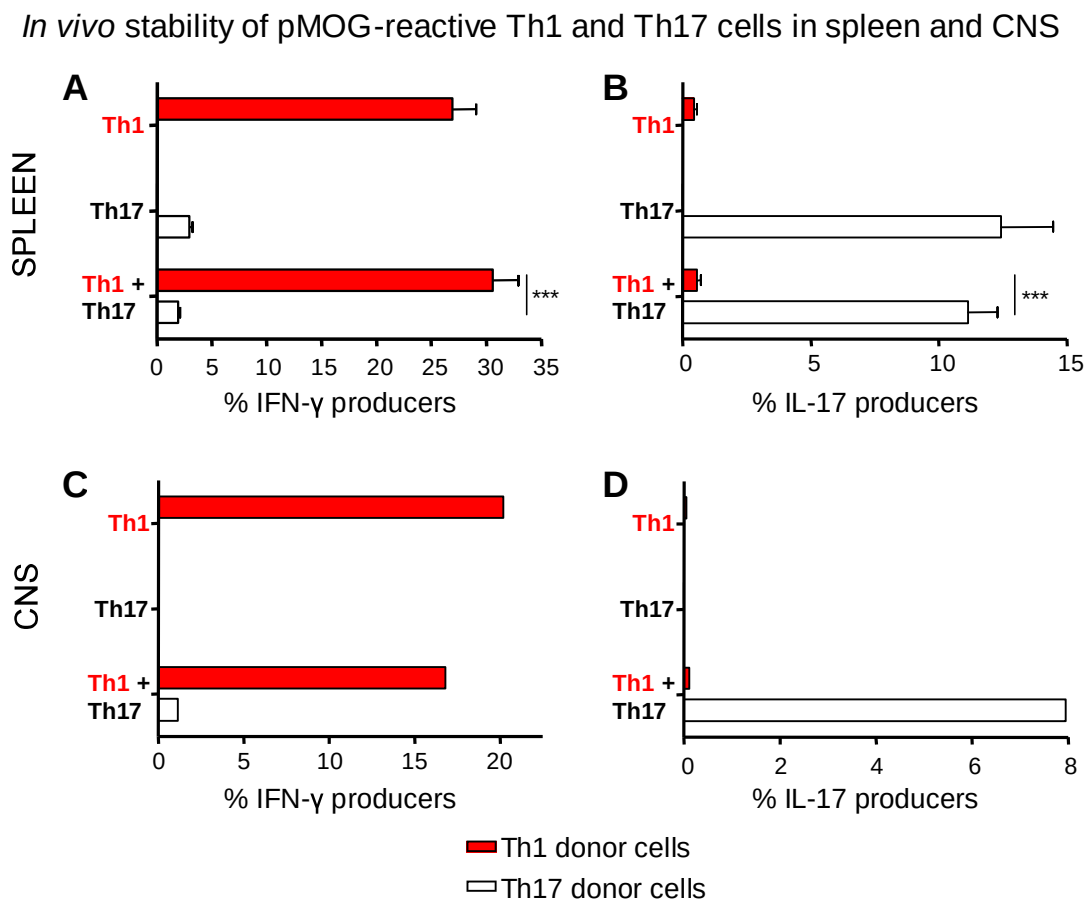

Figure 3.6 pMOG-reactive Th1 and Th17 cells remain phenotypically stable and produce IFN-γ and IL-17 respectively *in vivo*.

Gated on the $CD4^+$ donor population, quantitative representation of the percentage of **A,** IFN-γ^+ and **B,** IL-17^+ cells in the spleen; and the percentage of **C,** IFN-γ^+ and **D,** IL-17^+ cells in the CNS showing the Th1 donor cells (**red bar**) and Th17 donor cells (**clear bar**) after an over-night re-stimulation in the presence of 20 μg/ml pMOG. In the spleen significantly more Th1 cells were IFN-γ^+ compared to Th17 cells (*** p=0.0006), and significantly more Th17 cells were IL-17^+ compared to Th1 cells (*** p=0.0006). Statistics performed using Mann Whitney test ('*' represents * $p<0.05$; ** $p<0.01$; *** $p<0.001$). Error bars represent mean ± standard error. See Appendix 2 for flow cytometry gating strategy for donor cells and cytokine production. For the spleen, n=5 (Th1); 5 (Th17); 7 (Th1+Th17 co-transfer). For the CNS, samples were pooled for each group.

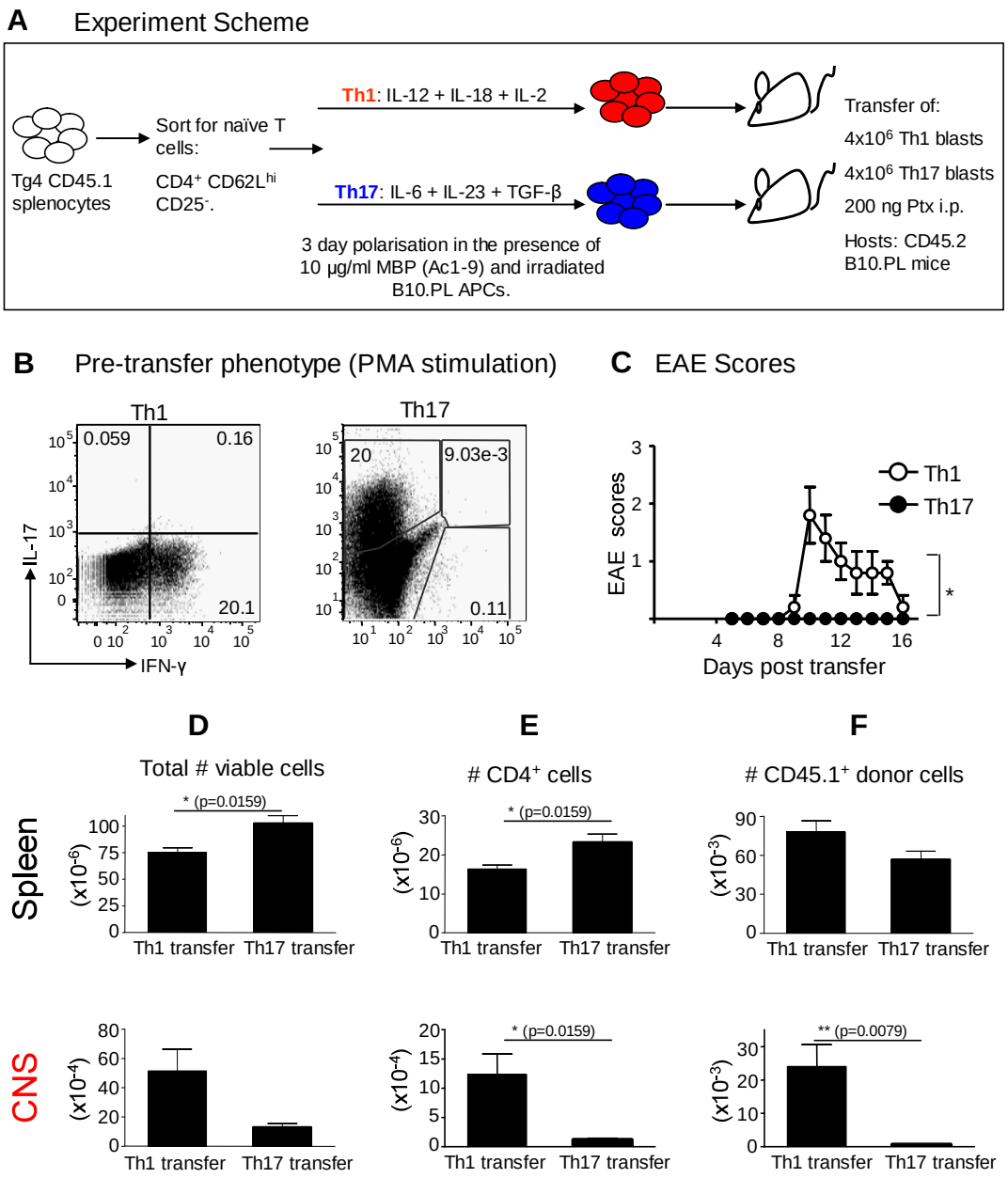

Figure 3.7 Tg4 Th1 cells induce disease whereas Tg4 Th17 cells do not

A, Experiment scheme; **B,** Gated on $CD4^+$ cells, pre-transfer phenotype of Tg4 Th1 (left) and Th17 (right), showing IFN-γ and IL-17 production, **C,** EAE scores after transfer of Tg4 Th1 (○) and Th17 (●) cells; **D,** total number of viable cells; **E,** absolute number of $CD4^+$ cells; **F,** absolute number of $CD45.1^+$ donor cells in the spleen (top) and CNS (bottom) in the Th1 and Th17 transfers. Disease incidence: Th1 (4/5) and Th17 (0/5). The proportion of mice with more severe EAE is higher in the Th1 transfer compared to the Th17 transfer (* p=0.0476). Statistics performed using Fisher's exact test comparing mice reaching a maximum EAE score of 1 and below, and, 2 and above. Error bars represent mean ± standard error. Statistical analyses for D-F performed using Mann Whitney test ('*' represents: * p<0.05; ** p<0.01; *** p<0.001). Disease incidence: Th1 (4/5); Th17 (0/5).

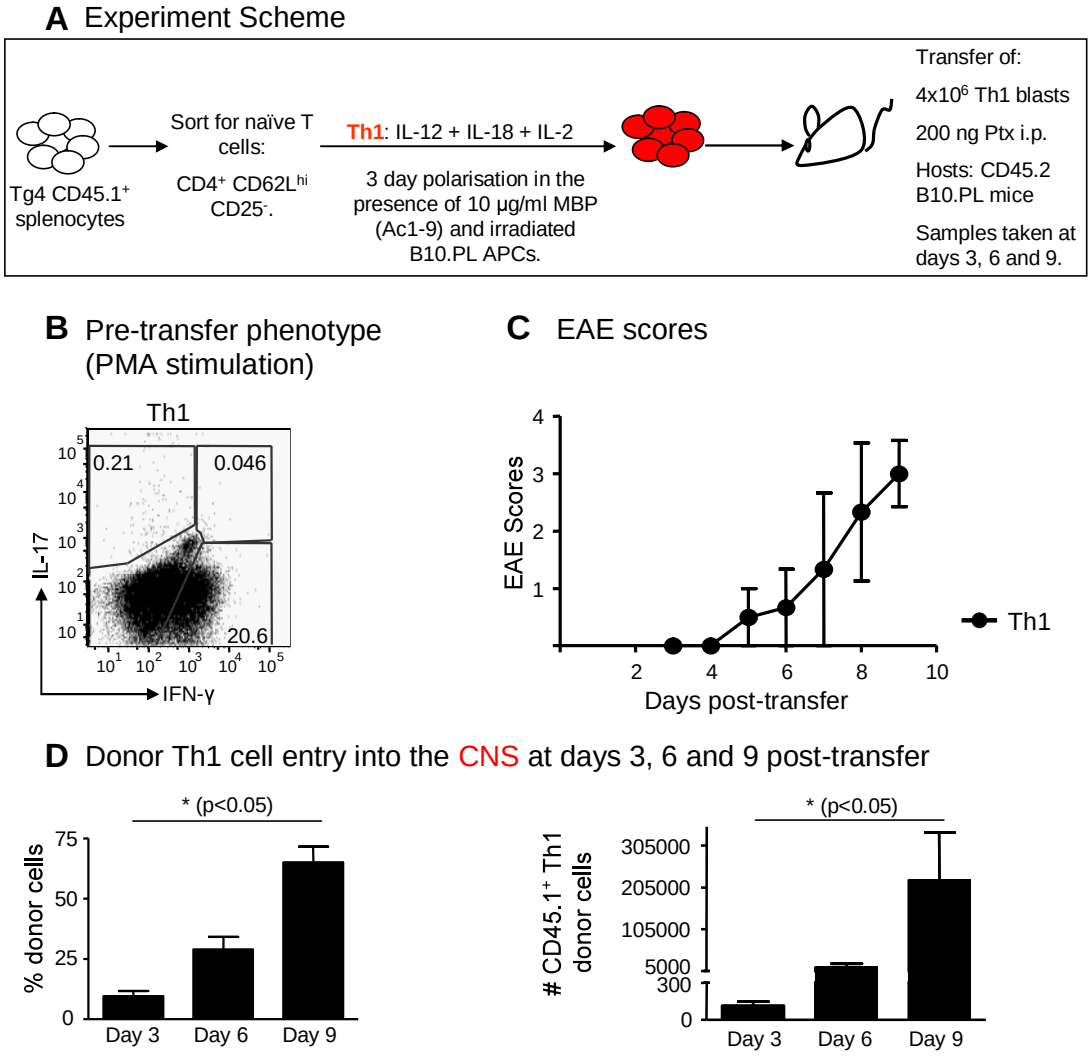

Figure 3.8 Tg4 Th1 cells can be located in the CNS as early as day 3 post-transfer.

A, Experiment scheme; **B,** Pre-transfer phenotype of Tg4 Th1 cells after PMA re-stimulation, gated on $CD4^+$ cells showing IFN-γ and IL-17 production; **C,** EAE scores after transfer of Tg4 Th1 cells (●); **D,** Percentage (left) and absolute number (right) of $CD45.1^+$ donor Th1 cells in the CNS at days 3, 6 and 9 post-transfer. Disease incidence: Th1 (3/3) (considering only mice taken through to day 9 post-transfer). Significantly higher percentage and absolute number of donor Th1 cells in the CNS between day 3 and day 9 post-transfer. Statistics performed using Kruskal Wallis test (KW p=0.0273) and a Dunn's multiple comparison post test. Error bars represent mean ± standard error. See appendix 3 for flow cytometry gating strategy for donor T cells in the CNS. Time-course of donor cell entry into the CNS representative of one experiment.

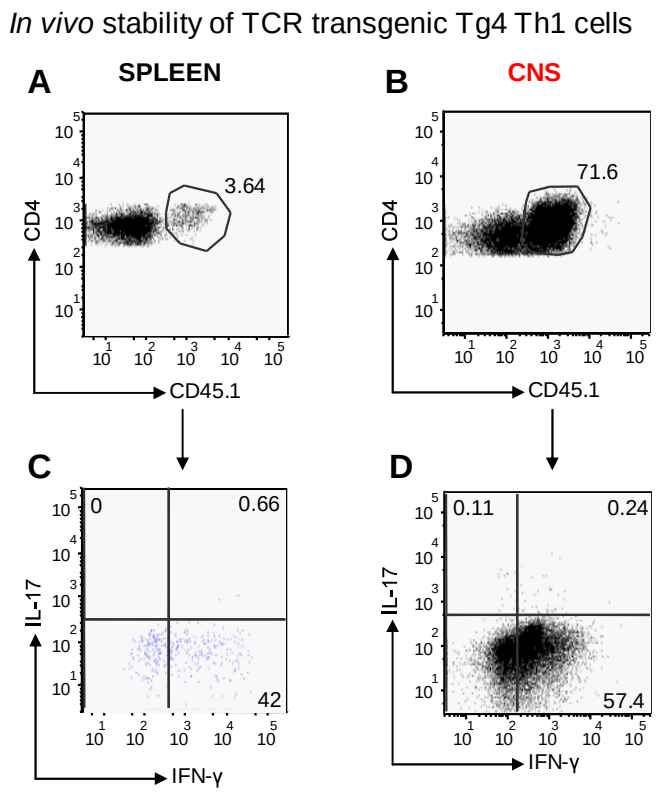

Figure 3.9 *In vitro* polarised Tg4 Th1 cells appear stable *in vivo*.

Intracellular cytokine staining on donor Th1 cells after an over-night re-stimulation with 20 µg/ml MBP (Ac1-9), and four hours incubation with Brefeldin A, Gated on $CD11b^-$ cells, presence of $CD4^+$ $CD45.1^+$ donor Th1 cells in the **A,** spleen and **B,** CNS; gated on the donor populations, production of IFN-γ and IL-17 in the **C,** spleen and **D,** CNS. Samples taken at day 9 post-transfer; 3 mice per group; showing pooled samples of each group. Gating determined on relevant isotype controls. Data representative of numerous Tg4 Th1 passive transfer experiments harvested at the peak of disease.

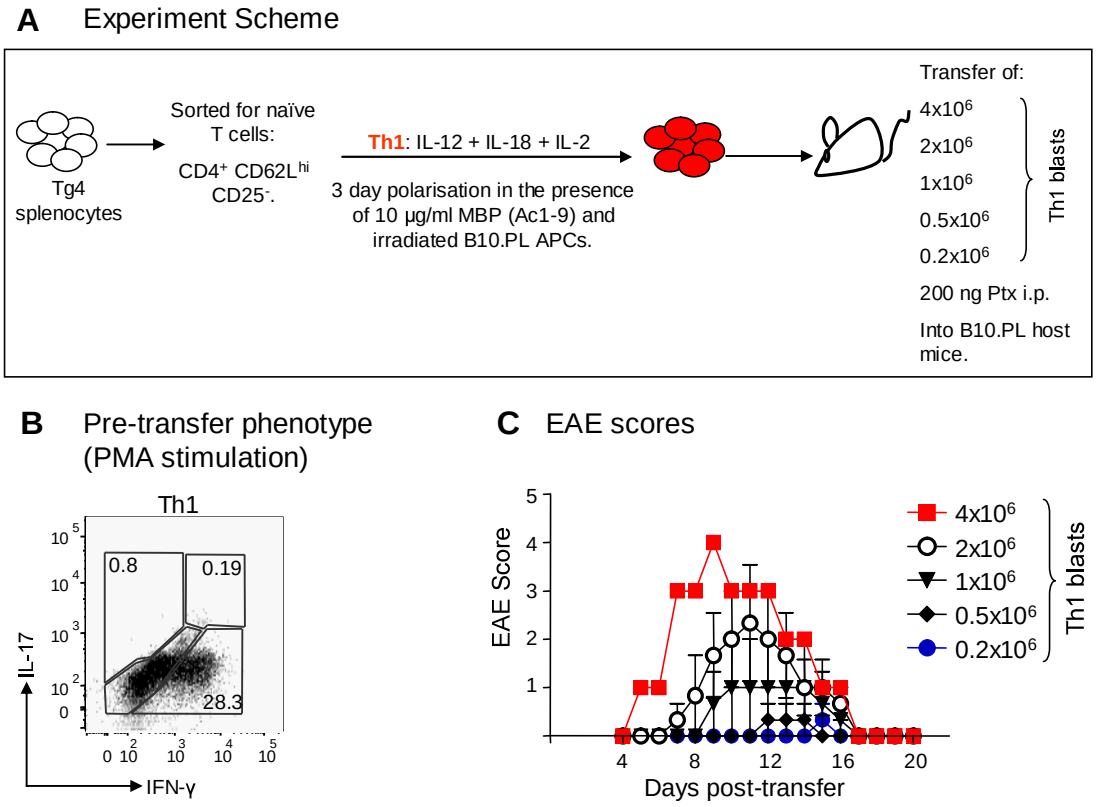

Figure 3.10 Tg4 Th1 cells induce EAE in a dose dependent manner.

A, Experiment scheme; **B,** Pre-transfer phenotype of *in vitro* polarised Tg4 Th1 cells, gated on $CD4^+$ cells showing IFN-γ and IL-17 production; **C,** EAE scores after transfer of $4x10^6$ (■), $2x10^6$ (○), $1x10^6$ (▼), $0.5x10^6$ (♦) and $0.2x10^6$ (●) Th1 blasts. Disease incidence: $4x10^6$ (1/1), $2x10^6$ (2/3), $1x10^6$ (1/3), $0.5x10^6$ (1/3), $0.2x10^6$ (1/3) Th1 blasts. No significant differences in EAE severity between the groups. Statistical analyses performed using the Fisher's exact test. See Appendix 4 for summary of results of statistical analyses. Error bars represent mean ± standard error. Dose-experiment representative of one experiment.

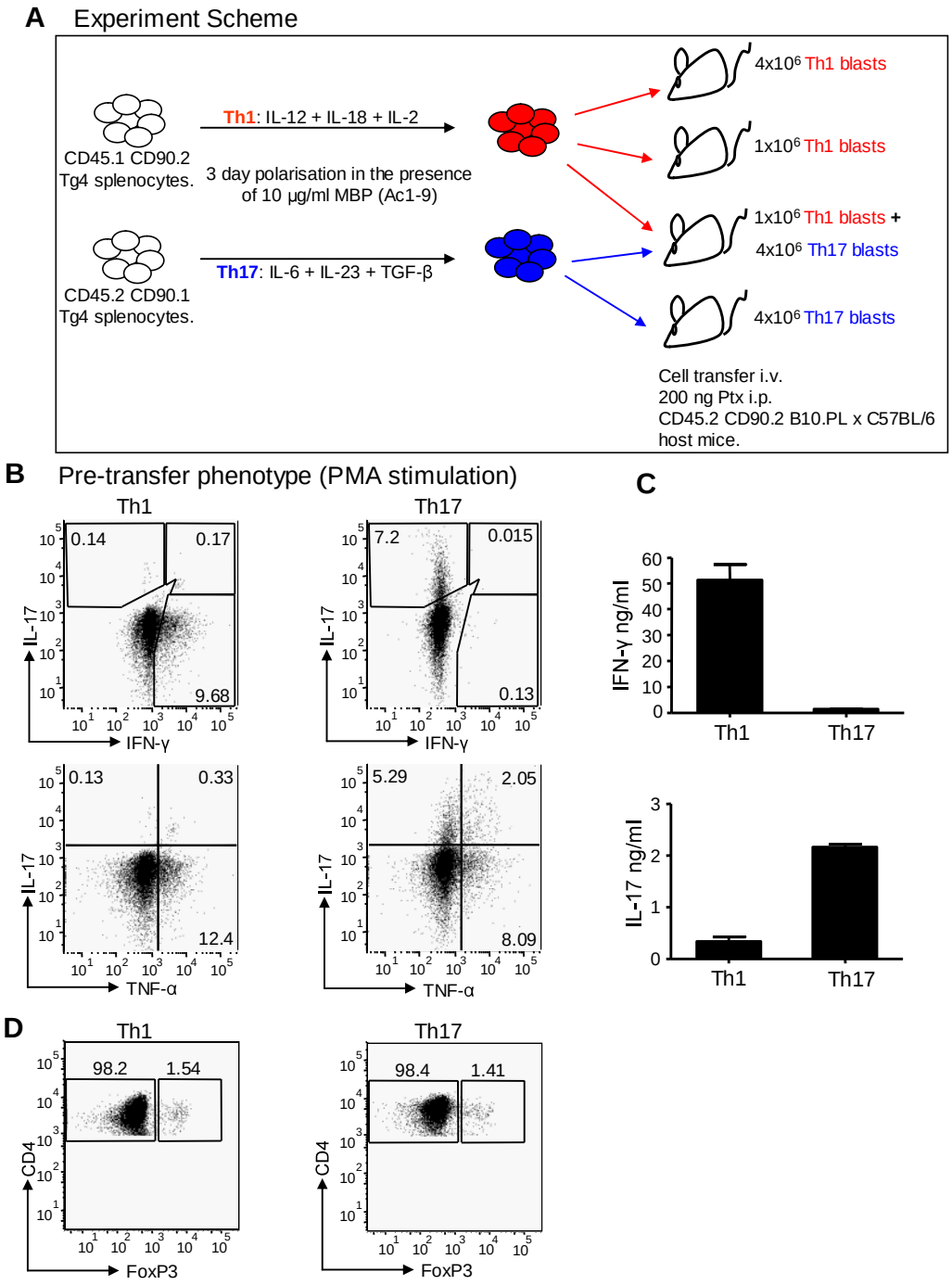

Figure 3.11 Transfer of sub-optimal numbers of Th1 cells together with high numbers of Th17 cells.

A, Experiment scheme; **B,** Pre-transfer phenotype of donor Tg4 Th1 (left) and Th17 (right) cells, gated on $CD4^+$ cells showing IFN-γ and IL-17 production (top) and TNF-α and IL-17 production (bottom); **C,** level of IFN-γ (top) and IL-17 (bottom) production in the Th1 and Th17 culture supernatant after 72 hours *in vitro* polarisation, as determined by ELISA; **D,** gated on $CD4^+$ cells, FoxP3 expression on Tg4 Th1 and Th17 cells prior to transfer. Error bars represent mean ± standard error. See Appendix 5 A for representative isotype controls for cytokine staining.

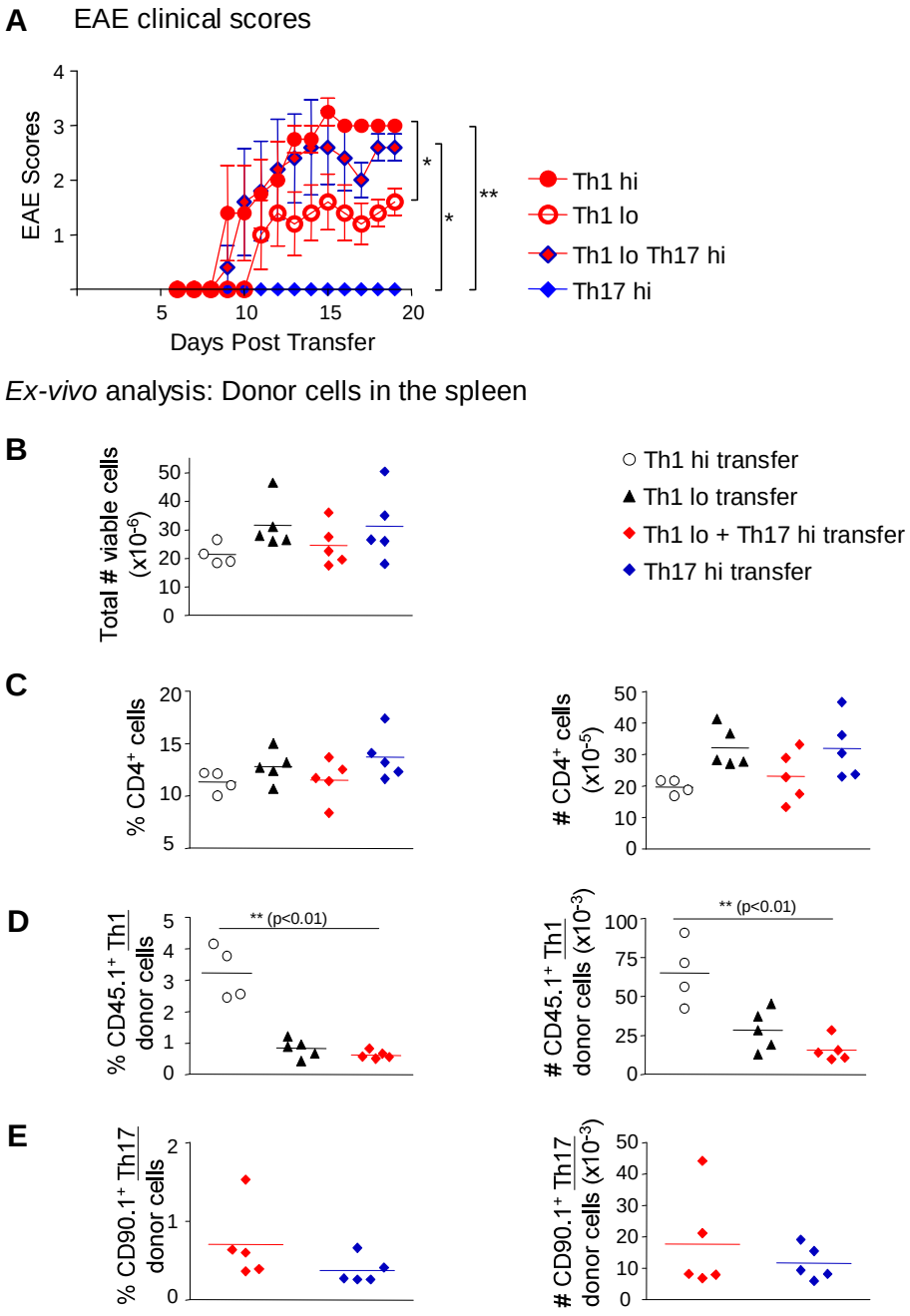

Figure 3.12 Tg4 Th17 cells are able to exacerbate disease to the same level as the Th1^{hi} transfer when co-transferred with a sub-optimal number of Th1 cells.

A, EAE scores after transfer of Th1^{hi} (4x10^6; ●), Th1^{lo} (1x10^6; ○), Th1^{lo} and Th17^{hi} (1x10^6 Th1 + 4x10^6 Th17; ◆) and Th17^{hi} (4x10^6; ♦) cells, Disease incidence: Th1^{hi} (5/5), Th1^{lo} (5/5), Th1^{lo} and Th17^{hi} (5/5) and Th17^{hi} (0/5); Location of the donor T cells in the spleen showing **B,** total number of viable cells; **C,** percentage and absolute number of CD4$^+$ cells; **D,** percentage and absolute number of CD45.1$^+$ Th1 donor cells; **E,** percentage and absolute number of donor CD90.1$^+$ Th17 cells for each transfer type; Th1^{hi} transfer (○), Th1^{lo} transfer (▲), Th1^{lo} Th17^{hi} transfer (♦), Th17^{hi} transfer (♦).

Underlined cells highlight the population being viewed. Statistical analyses performed using Kruskal-Wallis test and Dunn's Multiple comparison post test, or Mann Whitney test. Error bars represent mean ± standard error. The proportion of mice with severe EAE is significantly higher in the $Th1^{hi}$ transfer group compared to the $Th1^{lo}$ and $Th17^{hi}$ transfer (*p=0.0476 and *p=0.0079 respectively); as well as in the co-transfer group compared to the $Th17^{hi}$ group (*p=0.0476). Statistical analysis for EAE curve performed with the Fisher's exact test. See Appendix 5 B for details of statistical results. Tg4 Th1 and Th17 co-transfer experiment representative of one experiment.

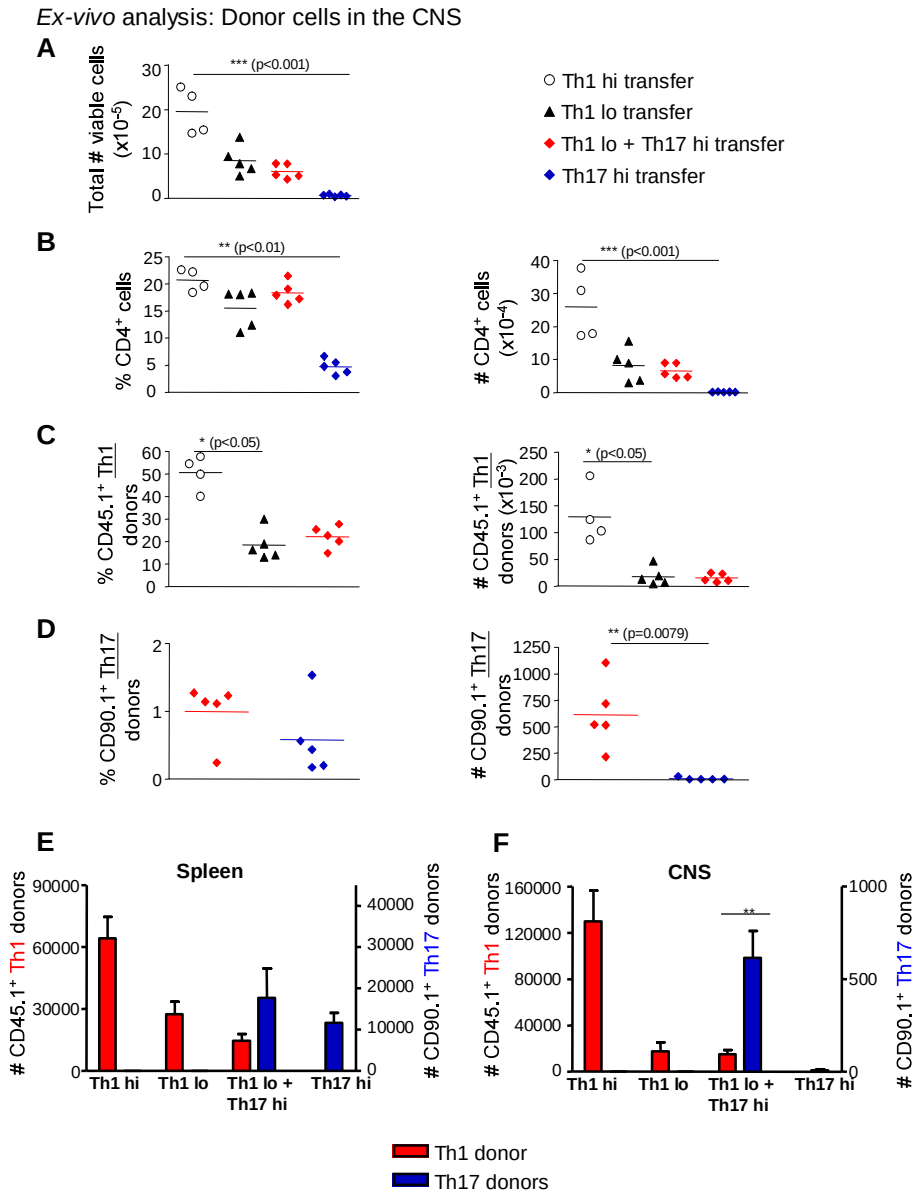

Figure 3.13 A significant number of Tg4 Th17 cells are able to enter the CNS when co-transferred with a sub-optimal number of Tg4 Th1 cells.

Ex-vivo analysis of location of donor cells in the CNS, **A,** total number of viable cells in the CNS; **B,** percentage and absolute number of $CD4^+$ cells; **C,** percentage and absolute number of $CD45.1^+$ Th1 donor cells; **D,** percentage and absolute number of $CD90.1^+$ Th17 cells in each transfer type, $Th1^{hi}$ transfer (○), $Th1^{lo}$ transfer (▲), $Th1^{lo}$ $Th17^{hi}$ transfer (♦), $Th17^{hi}$ transfer (♦); Summary of quantitative analysis of $CD45.1^+$ Th1 and $CD90.1^+$ Th17 donor cell in the **E,** CNS; and **F,** spleen showing Th1 donors (**red bar**) and Th17 donors (**blue bar**). Underlined cells highlight the population being viewed. Statistical analyses performed using Kruskal-Wallis test and Dunn's Multiple Comparison post test, and Mann Whitney test. Error bars represent mean ± standard error. See Appendix 5 C for flow cytometry gating analysis for donor cells in spleen and CNS. n=4 $Th1^{hi}$, n=5 $Th1^{lo}$, n=5 $Th1^{lo}$ $Th17^{hi}$, n=5 $Th17^{hi}$.

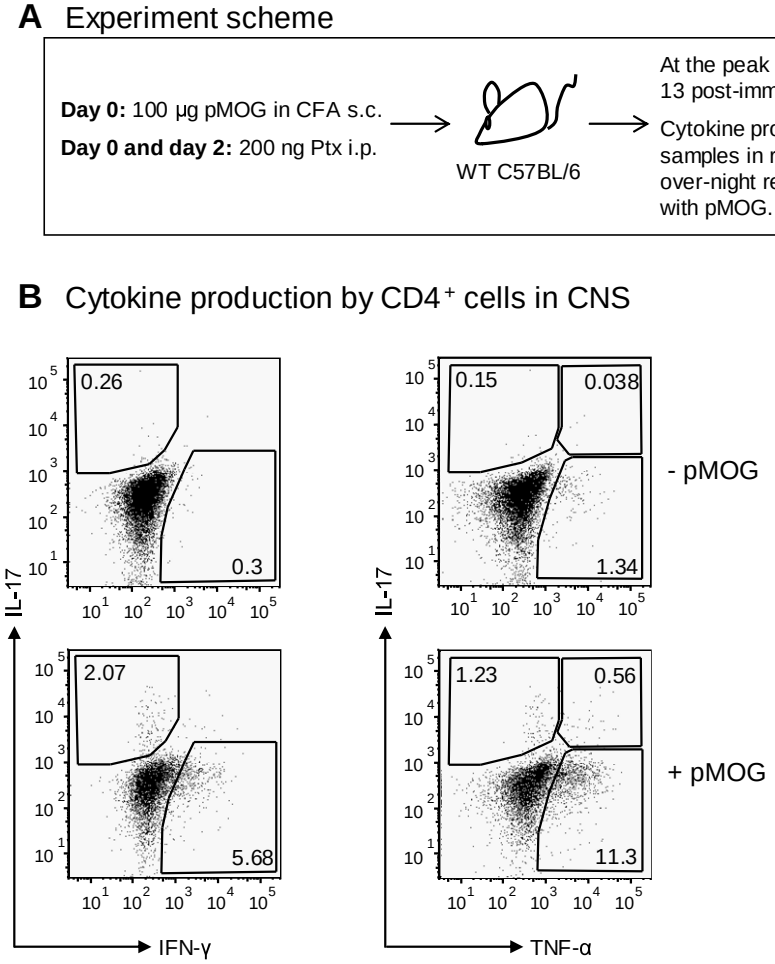

Figure 3.14 IFN-γ$^+$, IL-17$^+$ and TNF-α$^+$ CD4$^+$ T cells are in the CNS of EAE mice

A, Experiment scheme showing in induction of active EAE with pMOG/CFA immunisation of C57BL/6 mice; **B,** CNS samples taken at day 13 post-immunisation, gated on the CD4$^+$ cells showing IFN-γ, IL-17 and TNF-α after an over-night re-stimulation in the presence or absence of 20 µg/ml pMOG. Pooled samples from 5 mice.

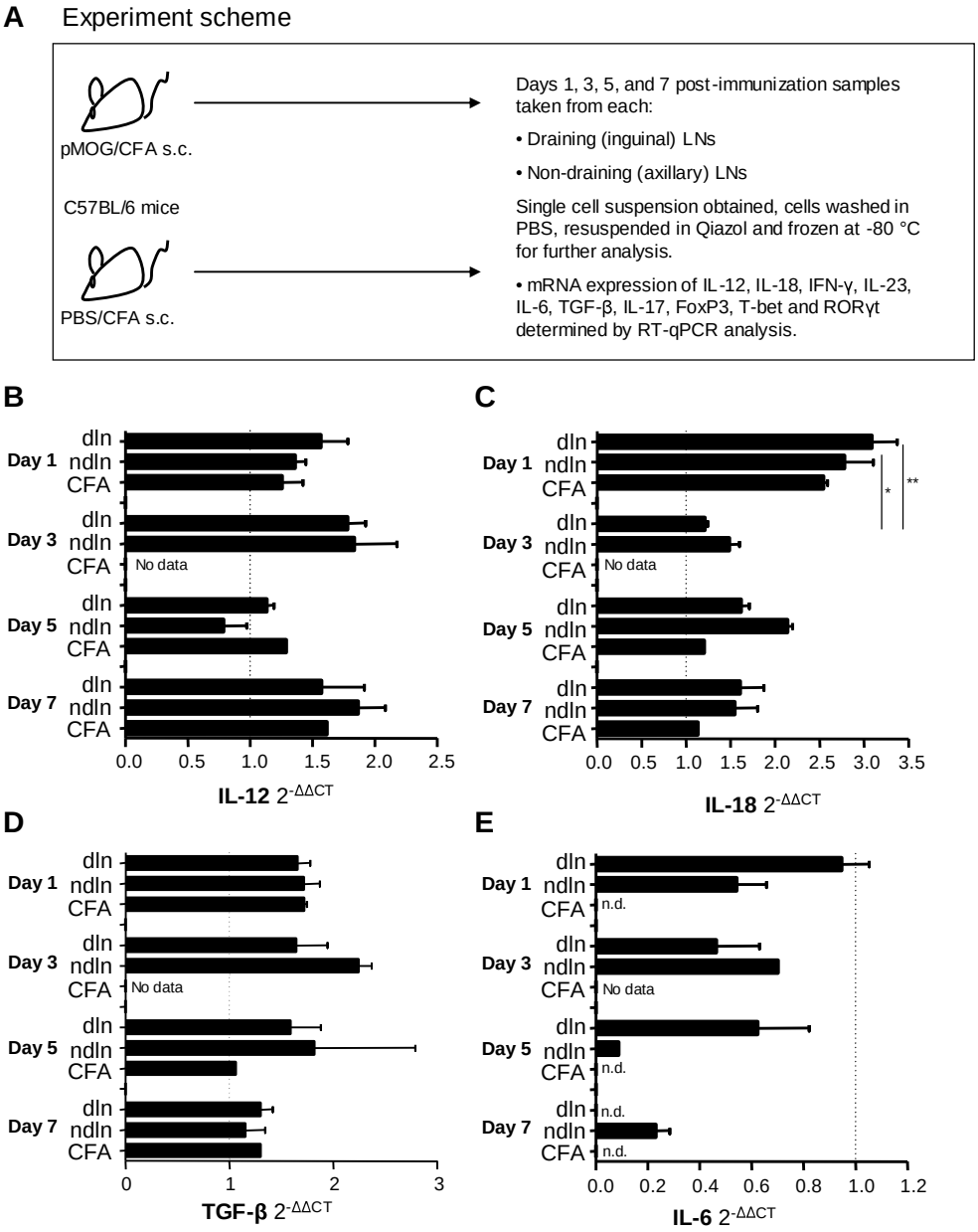

Figure 3.15 Levels of mRNA expression of IL-12, IL-18, TGF-β and TNF-α after CFA immunisation with/without pMOG, as compared to a non-immunised control

A, Experiment scheme; Changes in relative mRNA levels at days 1, 3, 5 and 7 post-immunisation for **B,** IL-12; **C,** IL-18; **D,** TGF-β; and **E,** IL-6 in draining lymph nodes (dln), non-draining lymph nodes (ndln) and CFA only controls (CFA) as compared to non-immunised naïve cells and to the house keeping gene, HPRT. Dotted line represents the control $2^{-\Delta\Delta CT} = 1$, which corresponds to no change in gene expression. Four pMOG/CFA mice per day, one CFA only control mouse per day, one non-immunised control per day. Error bars represent the mean ± standard error. Statistical analyses performed using Kruskal-Wallis test and Dunn's Multiple Comparison post test. Representative of one experiment.

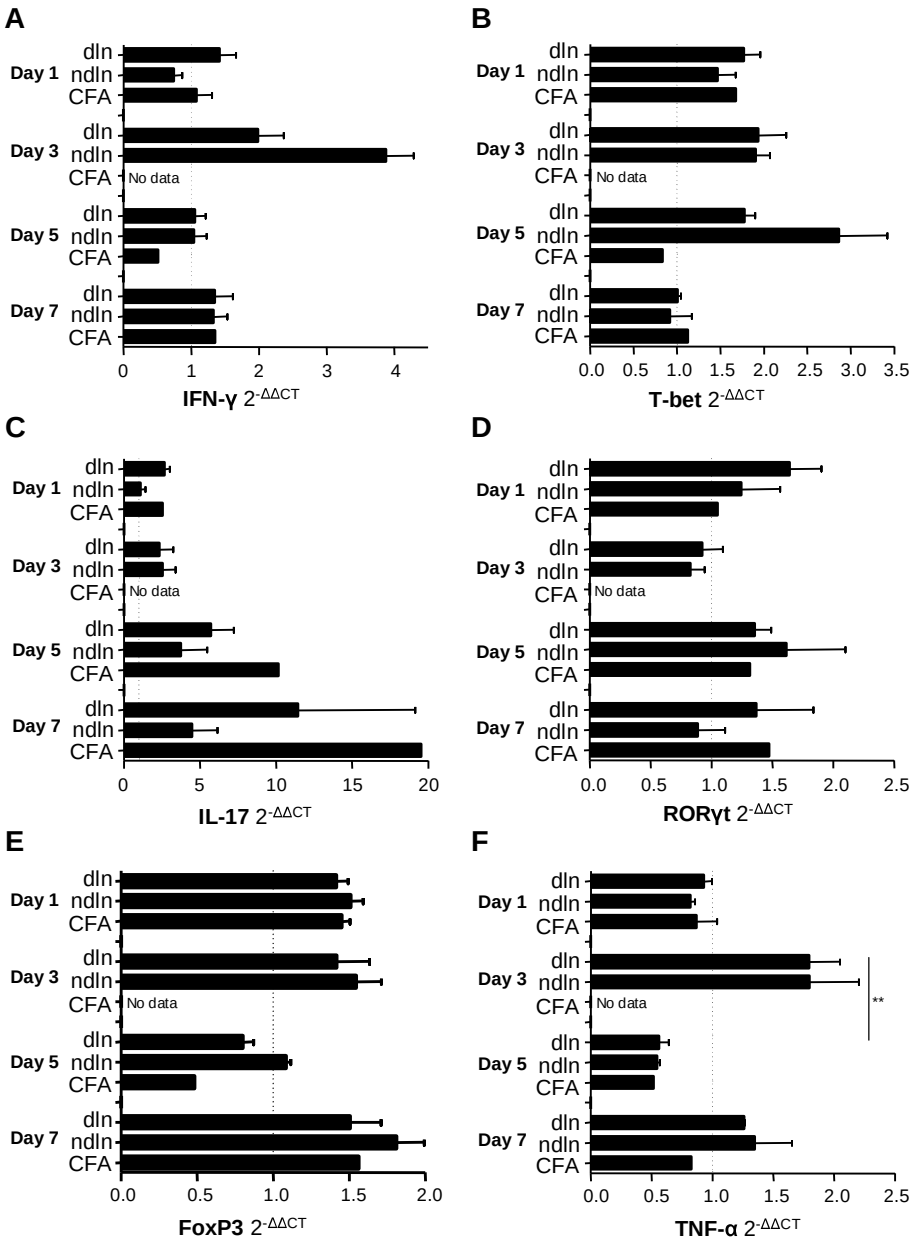

Figure 3.16 Level of mRNA expression for IFN-γ, IL-17, IL-17, T-bet, RORγt, FoxP3 and IL-6 after CFA immunisation with/without pMOG, as compared to a non-immunised control

Changes in relative mRNA levels at days 1, 3, 5 and 7 post-immunization for **A,** IFN-γ; **B,** T-bet; **C,** IL-17; **D,** RORγt; **E,** FoxP3 and **F,** TNF-α in the draining lymph nodes (dln), the non-draining lymph nodes (ndln) and CFA only controls (CFA) as compared to the non-immunised naïve cells and to the house keeping gene, HPRT. Dotted line represents the control $2^{-\Delta\Delta CT} = 1$, which corresponds to no change in gene expression. Four pMOG/CFA mice per day, one CFA only control mouse per day, one non-immunised control per day. Error bars represent the mean ± standard error. 'n.d.' indicates 'not detected'. Statistical analyses performed using Kruskal-Wallis test and Dunn's Multiple Comparison post test. Representative of one experiment.

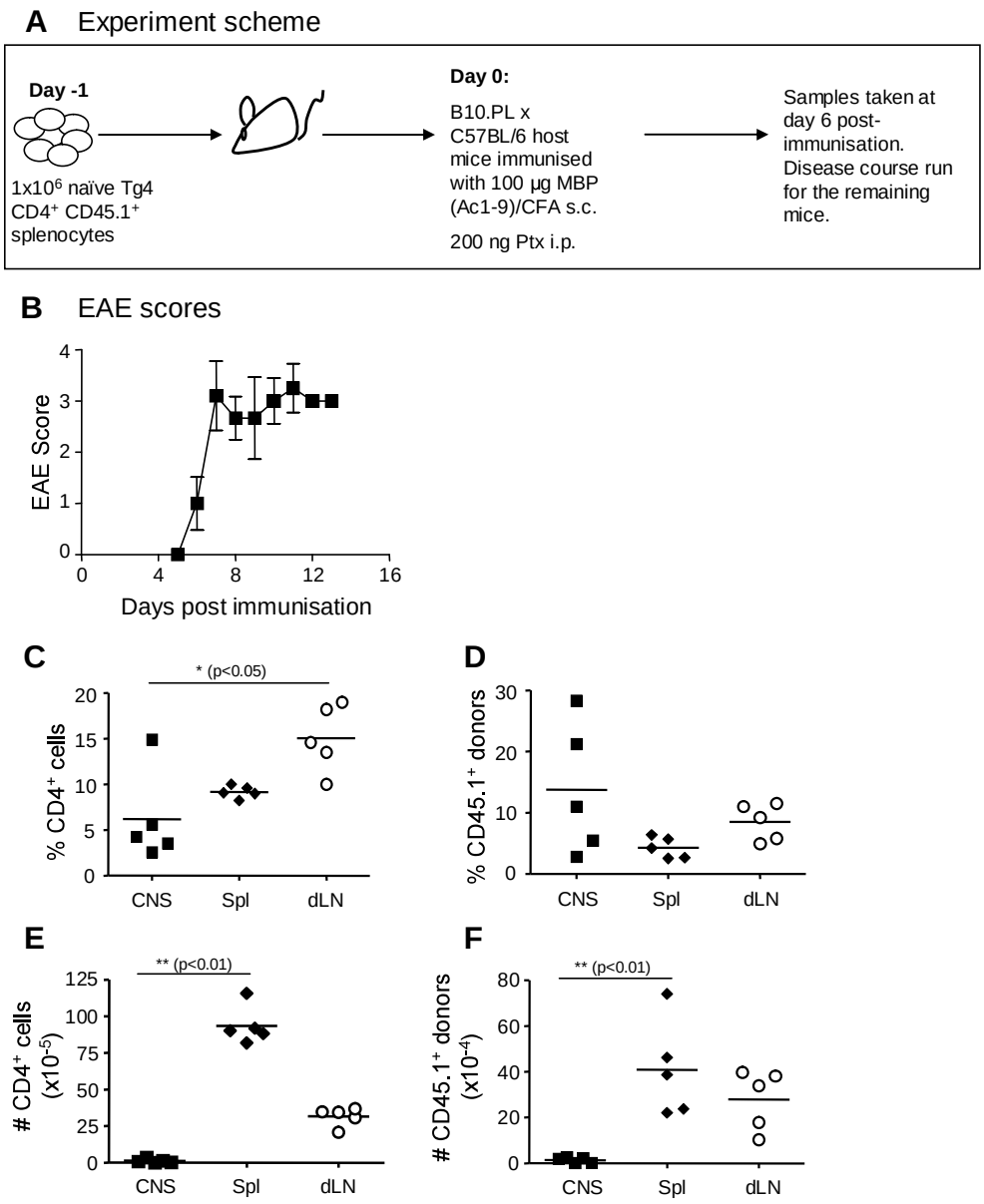

Figure 3.17 Phenotype of naïve Tg4 cells polarised *in vivo* after MBP (Ac1-9)/CFA immunisation.

A, Experiment scheme; **B,** Mean clinical EAE scores after disease induction (disease incidence (10/10)); day 6 post-immunisation, **C,** percentage $CD4^+$ $CD11b^-$ cells in CNS, spleen and draining (inguinal) lymph nodes; **D,** percentage $CD45.1^+$ donor Tg4 cells in the CNS, spleen and draining (inguinal) lymph nodes; **E,** absolute number of $CD4^+$ cells in each tissue; **F,** absolute number of $CD45.1^+$ donor cells in each tissue; 5 mice per group, all mice taken were score zero of clinical EAE. Error bars represent mean ± standard error. Statistics performed using Kruskal-Wallis test and Dunn's Multiple Comparison test (*p<0.05; **p<0.01; ***p<0.001). (KW p values: *p=0.0210 (C); **p=0.0019 (D); **p=0.0068 (E)). See Appendix 6 A for flow cytometry gating strategy for donor T cells. Representative of two experiments.

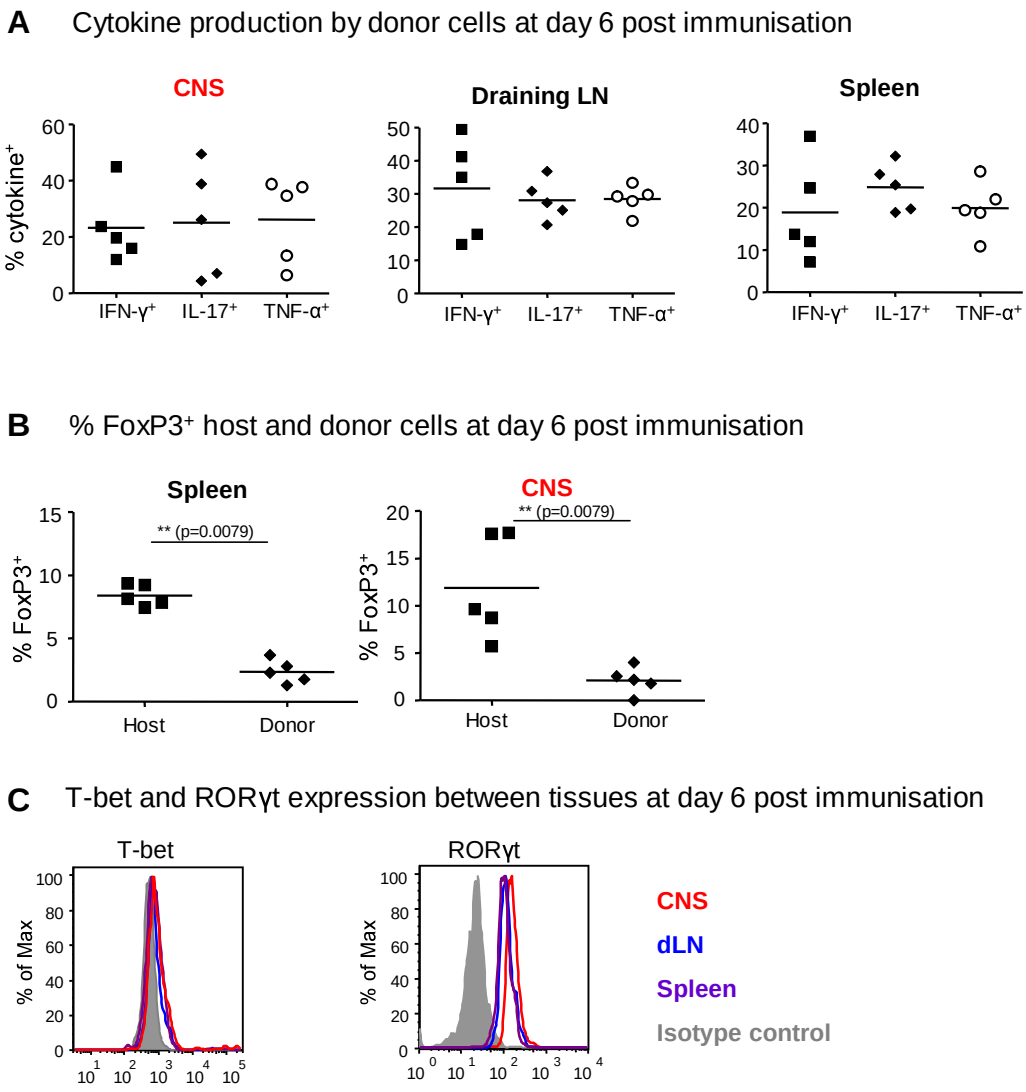

Figure 3.18 No indication of a differential skewing towards a Th1 or Th17 phenotype of the previously naïve Tg4 T cells at day 6 post immunisation.

Gated on $CD4^+$ $CD45.1^+$ donor Tg4 cells **A,** percentage IFN-γ^+, IL-17^+ and TNF-α^+ in the CNS (left panel), draining (inguinal) lymph node (middle panel) and spleen (right panel); **B,** percentage $FoxP3^+$ cells in the host and donor populations in the Spleen (left panel) and CNS (right panel); **C,** T-bet and RORγt expression on donor Tg4 cells between each tissue, showing CNS (**red**), draining lymph node (**blue**) and spleen (**purple**) and isotype control (**grey fill**), representative of 5 mice. No significant difference in cytokine production in each tissue, as determined by Kruskal Wallis test. Statistics for FoxP3 expression performed using Mann Whitney test ('*' represents: * $p<0.05$; ** $p<0.01$; *** $p<0.001$). See Appendix 6 B for flow cytometry gating strategy for donor cell cytokine production. n=5 mice.

4 Migration of encephalitogenic T cells into the central nervous system for the induction of EAE

4.1 Introduction

As demonstrated in Chapter 3, Th1 cells are readily able to infiltrate the CNS across the BBB and induce EAE (O'Connor et al., 2008). This ability of Th1 cells to home to the CNS has also been demonstrated in MS, in which Th1 cells are readily found in the CSF and within the CNS lesions of MS patients (Teleshova et al., 2002). Results in Chapter 3 showed within our passive transfer models, that Th17 cells have a poor ability to cross the BBB and induce disease. A simple explanation for the inability of Th17 cells to enter the CNS and induce disease, would be the lack of expression of one or more of the chemokine receptors, adhesion molecules or selectins that are involved in the entry of T cells across the BBB.

4.1.1 Aims

This observation allowed the comparison of pathogenic (Th1) cells with non-pathogenic (Th17) cells in terms of the cell surface phenotype. The aims were a) to identify molecules that were specifically expressed (or expressed at higher levels) on Th1 cells, and b) to block the function of these molecules, and assess the impact of this blockade on pathogenic activity using the passive transfer of Tg4 cells.

The establishment of disease by the accumulating Th1 cells in the CNS presumably led to increased permeability of the BBB, resulting in the further influx of more Th1 cells and other inflammatory cells, including Th17 cells into the CNS. The mechanisms involved in entering the non-inflamed CNS compared to the inflamed CNS, once disease is established, are presumably very different. It is therefore important to determine what allows these ‘pioneer’ Th1 cells across the normal ‘non-inflamed’ BBB before inflammation is established.

The CNS was long considered to be an immune privileged site (Barker and Billingham, 1977) with the BBB controlling the entry of T lymphocytes into the brain parenchyma. Wekerle and colleagues hypothesised that under normal physiological conditions, activated immune cells enter the CNS for the purpose of immune surveillance (Wekerle et al., 1986; Hickey, Hsu and Kimura, 1991). However during inflammatory conditions, seen with bacterial or viral infection, there is a large influx of lymphocytes across the BBB and into the CNS.

T lymphocyte entry to the brain parenchyma is a complex process involving numerous different molecules, including selectins, chemokine receptors, adhesion molecules and matrix metalloproteinases (reviewed in (Prendergast and Anderton, 2009)). In general there are thought to be four main steps for leukocyte trans-endothelial migration across the BBB: 1) an initial low-affinity contact between the lymphocyte and the endothelium is made resulting in the tethering of the lymphocyte and this facilitates their rolling along the endothelial cell surface. This initial process is mediated by selectins on the endothelial surface binding to glycosylated ligands on the lymphocyte surface (Engelhardt, 2008); 2) chemokines activate their respective chemokine receptors on the surface of the lymphocytes resulting in induction of G protein-linked intracellular signals. These signals lead to the activation of integrins on the lymphocyte surface, which then alter their conformation from the low affinity to the high affinity state; 3) integrins are then able to interact with their respective ligands on the surface of the endothelium, providing firm adhesion or complete arrest of the lymphocyte on the endothelium surface; 4) the lymphocyte extravasates across the endothelial layer into the perivascular space. To enter the brain parenchyma or spinal cord, the lymphocyte navigates through the tight junctions of the BBB or the blood-spinal cord barrier respectively. Due to the numerous different molecules involved in these processes and the various barriers in place, immune cell entry is kept to a minimum under normal physiological conditions.

Naïve T cells have a $CD44^{lo}$ $CD62L^{hi}$ phenotype. On activation, they downregulate molecules required for lymph node entry (e.g. CD62L and CCR7) (von Andrian and Mempel, 2003; Klinger et al., 2009) and upregulate molecules required for

lymphocyte migration to non-lymphoid tissues or the site of inflammation. Also on activation, $CD4^+$ T cells differentiate into particular effector sub-types, namely Th1, Th2, Th17 or Treg cells depending on the cytokine milieu in the surrounding environment. Each Th subset expresses a distinct pattern of adhesion molecule and chemokine receptor expression allowing them to have differing abilities to enter the CNS or other non-lymphoid tissues according to their homing molecule expression profile.

In general, human Th1 cells are thought to express CCR5 and CXCR3 whereas human Th2 cells express CCR3 and CCR4 (Bonecchi et al., 1998). This view correlated with MS being viewed as a Th1-mediated disease due to a large influx of $CCR5^+$ $CXCR3^+$ T cells in MS lesions, as well as increased expression of MIP-1α and IP-10, their respective ligands (Balashov et al., 1999). Human Th17 cells have been found to have a $CCR2^+$ $CCR5^-$ phenotype (Sato, Aranami and Yamamura, 2007) and to express CCR6 (Annunziato et al., 2008). The expression of different chemokine receptors on the surface of T cell subsets, suggests a role in differential migration of the cells to specific tissues and sites of inflammation.

Chemokine receptors or adhesion molecules involved in the entry of T cells into the CNS are attractive targets for therapeutics for CNS autoimmune disease. Preventing the entry of the inflammatory T cells into the CNS would prevent the induction of CNS inflammation, subsequent demyelination and lesion formation. The feasibility of targeting migration molecules for therapeutics has been demonstrated by the development of Natalizumab (Tysabri®) a monoclonal antibody against integrin α4β1 for the treatment of RRMS. It was initially found that EAE was both suppressed and reversed after the administration of a monoclonal antibody to alpha 4 integrin (Kent et al., 1995) in the guinea pig. This then novel therapeutic was translated into the clinic. Natalizumab is used for the treatment of both MS and Crohn's disase and it is found to decrease relapses and prevent the formation of new lesions within the CNS of RRMS patients. The efficacy of Natalizumab shows the potential for targeting other migratory molecules for the inhibition of inflammatory T cell entry into the CNS for the treatment of MS. However, α4-integrins are expressed

on all leukocytes and therefore Natalizumab is a fairly non-specific treatment with the development of PML as its major risk. Identification of a homing molecule specifically expressed on encephalitogenic T cells would provide greater specificity for therapeutics and fewer side effects or risks.

4.1.2 Approach

First, phenotypic differences between pathogenic Th1 and non-pathogenic Th17 cells were determined. Once particular molecules of interest were identified, their function was blocked *in vivo*. The Tg4 passive transfer model was used to investigate the effect of blocking the homing molecules on EAE pathogenicity. To do this Tg4 splenocytes were taken and polarised towards a Th1 phenotype in the presence of polarising cytokines IL-12, IL-18 and IL-2 and in the presence of 10 μg/ml MBP (Ac1-9) for three days. Cells were then washed and transferred into host mice by intravenous injection with/without either pre-treatment of the cells, or *in vivo* administration of blocking molecules, dependent on the molecule being investigated.

4.2 Results

4.2.1 Th1 and Th17 cells have comparable levels of expression of α4β1-integrin

As blocking α4β1-integrin has been shown to have high efficacy in treating both EAE and MS, it was first verified whether *in vitro* polarised Th1 cells expressed higher levels of α4β1-integrin which could explain their ability to home to the CNS in comparison to Th17 cells. However, Tg4 Th1 cells did not have a higher expression level of α4β1-integrin than Th17 cells (Figure 4.1 A and B). In fact, neither population appeared to express α4β1-integrin above the isotype control. However, due to the absence of a positive control for the α4β1-integrin staining, it cannot be concluded from this data that neither population express α4β1-integrin.

4.2.2 Expression of chemokine receptors on Th1 and Th17 cells

The expression of a panel of chemokine receptors was verified on the *in vitro* polarised Th1 and Th17 cells. Expression of CCR2, CCR3 and CCR4 was absent on the Th1 and Th17 cells (Figure 4.2 A-C). Both Th1 and Th17 cells expressed CXCR3 to the same level (Figure 4.2 F). CCR5 was expressed to a higher level on the Th1 cells in comparison to the Th17 cells (Figure 4.2 D; n=3) although no statistical analyses were performed here.

4.2.3 Th1 cells express Th17-associated CCR6 at the protein level

By flow cytometry analysis both populations expressed the Th17-associated molecule CCR6 to the same extent (Figure 4.2 E). This observed expression of CCR6 on Th1 cells was surprising. In a second experiment, CCR6 expression was determined by flow cytometry analysis with the use of *in vitro* polarised Th2 cells as a negative control for CCR6 expression. $CD4^+$ Th2 cells did not express CCR6, whereas both $CD4^+$ Th1 and $CD4^+$ Th17 cells did (Figure 4.3 A, B). CCR6 mRNA expression was assessed on Tg4 Th1 and Th17 cells. Using Th0 cells as the control ($2^{-\Delta\Delta CT} = 1$), Th1 cells had a $2^{-\Delta\Delta CT}$ of 1.6 whereas CCR6 was highly upregulated on Th17 cells with $2^{-\Delta\Delta CT}$ of 14.7 as compared to the control, showing a nearly 15-fold upregulation (Figure 4.3 C). Th1 cells therefore appeared to express CCR6 at the protein level but have low mRNA CCR6 expression, perhaps indicating instability in the CCR6 mRNA in these cells, as discussed later.

4.2.4 Th1 cells express higher levels of CD62L and PSGL-1

Investigating other homing molecules, Th1 cells had higher expression levels of CD62L (Figure 4.4 B) and PSGL-1 (Figure 4.4 C) compared to Th17 cells.

In summary, the expression of CCR5, CD62L and PSGL-1 were observed to be upregulated on Th1 cells compared to Th17 cells and these were followed up in more detail through the use of blocking studies. CCR6 was expressed on both the Th1 and Th17 cells at the protein level. In the same way, α4β1-integrin expression was comparable on Th1 and Th17 cells. These two molecules were not followed up in detail as they did not exhibit differential expression on the two *in vitro* polarised populations, and the aim was to identify phenotypic differences that would account for the ability of the pathogenic Th1 cells to enter the CNS and not the Th17 cells.

4.2.5 Blocking CCR5 signalling using Tak779 has no effect on EAE induction by Tg4 Th1 passive transfer

As shown in Figure 4.2 *in vitro* polarised Th1 cells had a higher expression of CCR5 compared to Th17 cells. The next question to answer was whether CCR5 was necessary for the entry of Th1 cells into the CNS to induce disease. This was done using Tak779, a low molecular weight non-peptide inhibitor that binds CCR5, CXCR3 and CCR2, thereby inhibiting the signalling through these molecules (Baba et al., 1999; Gao et al., 2003). It is thought to bind CCR5-expressing cells and prevent the binding of its ligand CCL5 (RANTES).

Tg4 Th1 blasts were transferred into hosts. The following day, and every second day thereafter, each host mouse was treated with 50 μg Tak779 (equivalent to approximately 2 mg/kg), or PBS, by intravenous injection (Figure 4.5 A). The transferred cells had a Th1 phenotype (Figure 4.5 B and C) and had a $CD4^+$ $CCR5^+$ population as compared to the isotype control (Figure 4.5 D).

There was no significant difference in the disease induction, severity or incidence between the Tak779 and PBS treated groups (Figure 4.6). A pre-clinical sample of mice from both groups was taken at day 7 post-transfer. There was no significant difference in the total number of cells in the spleen or CNS at this time point (Figure 4.7 A). There was also no significant difference in the number of $CD4^+$ cells or $CD45.1^+$ Th1 donor cells in the spleen or CNS (Figure 4.7 B and C) indicating the

treatment of the mice with Tak779 had had no effect on the entry of the donor Th1 cells into the CNS.

4.2.6 CD62L is not required for the induction of EAE or the entry of Tg4 Th1 cells into the CNS

CD62L is known to be required for the entry of naïve T cells into the lymphoid organs but it is not clear if it is required for the entry of Th1 cells into the CNS due to conflicting data in the literature. To determine if it was required for the induction of EAE here, Tg4 Th1 cells were transferred into host mice together with either an infusion of either PBS or the anti-CD62L monoclonal antibody, Mel-14. Every two days post-transfer, the mice received a further dose of Mel-14 or PBS (Figure 4.8 A). The pre-transfer analysis of the donor cells showed their Th1 phenotype with high IFN-γ^+ and TNF-α^+ cells and negligible numbers of IL-17^+ cells (Figure 4.8 B) and upregulated expression of T-bet (Figure 4.8 C). Importantly the Th1 cells were $CD62L^{hi}$ (Figure 4.8 D) and when treated with Mel-14 *in vitro* the cells were shown to have lower levels of CD62L expression, confirming the binding of the antibody (Figure 4.8 D).

Administration of the Mel-14 had no effect on the induction of EAE by the Tg4 Th1 cells (Figure 4.9). At a pre-clinical time-point, four mice were sacrificed from each group to determine the effect of the administration of Mel-14 on the location of the donor Tg4 Th1 cells within the host mice. There was no difference in the total number of cells or $CD4^+$ cells found in the lymph nodes after *in vivo* treatment with Mel-14 (Figure 4.10 A). However, there were significantly lower numbers of Th1 donor cells within the lymph node after treatment with Mel-14 (Figure 4.10 A). In contrast, the administration of Mel-14 had no effect on the entry of donor Tg4 Th1 cells into the spleen or CNS (Figure 4.10 B and C). This result was representative of two similar experiments.

4.2.7 Both Th1 and Th17 polarised cells express the enzyme C2GnT-I to comparable levels

PSGL-1 is a 240 kD homodimer that binds E-, P- and L-selectin when glycosylated. The two key glycosyltransferases and sialyl transferases required for the expression of functional PSGL-1 are core 2 β-1.6-N-acetyl glucosaminyltransferase (C2GnT-I) and α-(1,3)-fucosyltransferase-VII (FucT-VII). Mice that lack either enzyme have been shown to have impaired binding of PSGL-1 to P-selectin and this results in reduced rolling of the lymphocytes in venules *in vivo* (Smithson et al., 2001; Sperandio et al., 2001).

The mRNA expression of C2GnT-I was determined on Th1, Th17 and Th0 cells. To first confirm the phenotype of the cells, T-bet and RORγt expression was determined on the cells by RT-qPCR. Both Th0 and Th1 cells were found to have upregulated expression levels of T-bet compared to Th17 cells (Figure 4.11 A) whereas Th17 cells had highly upregulated levels of RORγt expression (~25 fold more than Th0 cells) (Figure 4.11 B). Th1 cells were found to have 2.5 fold higher expression of C2GnT-I than Th0 cells and Th17 cells had roughly similar levels at 2.2 fold higher than the control cells (Figure 4.11 C). Therefore the Th1 and Th17 cells appear to have comparable expression levels of C2GnT-I, indicating that both cell populations can express functional PSGL-1.

4.2.8 Pre-treatment of Tg4 Th1 cells with anti-PSGL-1 antibody results a delay in EAE induction

The 4RA10 monoclonal antibody against PSGL-1 was used to investigate whether blocking the function of PSGL-1 on Tg4 Th1 cells prior to their transfer would have an effect on their ability to induce EAE by passive transfer. After polarisation the cells were washed and treated with 10 μg/ml anti-PSGL-1 (or PBS) for 1 hour on ice (Figure 4.12 A) and subsequently transferred into host mice. On the day of transfer, the cells showed a Th1 phenotype with high proportions of IFN-γ^+ cells and negligible IL-17^+ cells (Figure 4.12 B). In addition, the majority of the $CD4^+$ cells in the Tg4 Th1 population transferred were PSGL-1^+ (Figure 4.12 B).

Pre-treatment of donor cells with anti-PSGL-1 led to a significant delay in the induction of EAE (Figure 4.13). However, once disease had developed, the incidence and severity were comparable to the PBS-treated control group. A Mann Whitney test was used here in order to determine if there was a significant difference between the mean day of disease induction between the two groups. A Fisher's Exact test would not have been appropriate here as there was no observed difference in disease severity.

There was no significant difference in the total number of cells in the spleen, or in the number of $CD4^+$ cells or $CD45.1^+$ donor cells in the spleen at day 4 post-transfer (Figure 4.14 A-C, left). In the CNS, there was a trend towards fewer total numbers of cells in the anti-PSGL-1 pre-treated group (Figure 4.14 A, right). The observed delay in disease induction correlated with fewer $CD4^+$ T cells and $CD45.1^+$ Th1 donor cells in the CNS (Figure 4.14 B-C, right) at this pre-clinical time-point of disease. The trend of fewer donor Th1 cells in the CNS of the anti-PSGL-1 treated cells was consistent over 3 further experiments; however it did not reach significance. Blocking the function of PSGL-1 on Th1 cells prior to adoptive transfer therefore appeared to result in a delay in the induction of disease. These cells appeared to have an impaired ability to cross the BBB and enter the CNS as shown by the reduced number of donor cells in the CNS at an early pre-clinical time point.

4.2.9 *In vivo* blockade of PSGL-1 resulted in the abrogation of disease

As there was a promising trend towards fewer donor cells entering the CNS when they were pre-treated with anti-PSGL-1, it was next determined whether *in vivo* blockade of PSGL-1 would have a more profound affect on the induction of EAE. To do this, Th1 cells were pre-treated as above with 10 μg/ml anti-PSGL-1 for 1 hour prior to transfer. In addition, the host mice were treated with 25 μg anti-PSGL-1 in PBS, or PBS alone, on days 1, 3, 5, 7, 9, 11 post cell transfer (Figure 4.15 A-C).

The additional *in vivo* blockade of PSGL-1 completely abrogated the induction of EAE for the duration of treatment (Figure 4.16). The two PBS treated mice developed EAE, however the 3 mice treated with anti-PSGL-1 did not. On day 13 post-transfer (two days after the final antibody dose), one of the anti-PSGL-1 treated mice began to develop a mild course of disease (Figure 4.16).

To determine if this inhibition of disease was due to the inability of the donor Tg4 Th1 cells to enter the CNS, this experiment was repeated. Anti-PSGL-1, or control IgG1 antibody, was administered on days 0 (together with transferred cells), 2, 4 and 6 post-transfer (Figure 4.17 A). Importantly, the difference between this experiment and the one shown in Figure 4.15 is that these cells were not pre-treated with the blocking antibody prior to transfer.

Due to 2 mice dying on day 6 post-transfer a few minutes after the treatment with the anti-PSGL-1 (suggestive of an anaphylactic reaction) it was decided to end the experiment on day 7 post-transfer to determine if there was differential migration of the donor Tg4 Th1 cells between the two groups. In the spleen (Figure 4.17 B-D, left) and inguinal lymph nodes (data not shown) there was no significant difference in the total cell number between the two groups, or in the number of $CD4^+$ cells. There were significantly fewer $CD45.1^+$ Th1 donor cells in the spleen compared to the IgG1 control group (Figure 4.17 D, left). In the CNS there were significantly lower total cell numbers in the anti-PSGL-1 treated mice compared to the IgG1 treated mice (Figure 4.17 B, right). However, this difference in total cell number did not correlate with a difference in the number of total $CD4^+$ cells in the CNS (Figure 4.17 C, right), or with the number of $CD45.1^+$ donor Th1 cells (Figure 4.17 D right) between the two treatment groups. As PSGL-1 is also expressed on other cells, the effect of anti-PSGL-1 treatment on the presence of $CD11b^+$ cells was determined in the spleen and CNS. In the spleen the treatment with anti-PSGL-1 led to a significant reduction in $CD11b^+$ cells both as frequency and absolute number (Figure 4.18 A, B). However, anti-PSGL-1 had no effect on the number of $CD11b^+$ cells in the CNS (Figure 4.18 C, D). In summary, the treatment with anti-PSGL-1 led to fewer total lymphocytes in the CNS, but the number of Th1 donor cells was comparable to

control treated mice. Anti-PSGL-1 also surprisingly and very effectively reduced the number of $CD11b^+$ cells in the spleen.

4.2.10 Increasing the expression of PSGL-1 and CCR5 on Th17 cells does not confer pathogenic activity

As PSGL-1 expression was found to be lower on Th17 cells compared to Th1 cells, it was determined whether increasing the expression of PSGL-1 on the Th17 cells, by the addition of IL-12 to the polarising cytokine mix, would have an effect on PSGL-1 expression, and thereby the ability of Th17 cells to induce EAE. To do this, Tg4 splenocytes were polarised towards a Th1 or a Th17 phenotype, or towards a 'Th17+IL-12' phenotype (using IL-6, IL-23, TGF-β and IL-12 for this last condition) (Figure 4.19 A). After 3 days of polarisation the Th1 and Th17 polarised cells exhibited the anticipated Th1 and Th17 phenotypes (53% IFN-γ^+ and 23% IL-17^+ cells, respectively) (Figure 4.19 B). In addition to this the 'Th17+IL-12' cells had a similar cytokine profile to the Th17 cells (22% IL-17^+ (Figure 4.19 B)). The 'Th17+IL-12' cells only had a slight increase in IFN-γ^+ cells (from 0.5% to 1.8%) above the Th17 population (Figure 4.19 B). The Th17 cells expressed PSGL-1 to a lower level than the Th1 cells with a clear high and low PSGL-1^+ population (Figure 4.19 C). Interestingly the 'Th17+IL-12' cells expressed PSGL-1 to the same level as the Th1 cells (Figure 4.19 C). A slight increase in CCR5 expression on the Th17 cells in the presence of IL-12 was also observed (Figure 4.19 D).

Despite the increased expression of PSGL-1 on the 'Th17+IL-12' group of cells, this did not confer pathogenicity (Figure 4.20 A). The mice were sampled at day 20 post-transfer, as the Th1-transferred group were beginning to recover from disease. At this time-point, there was a significantly increased total number of cells observed in the CNS of Th1-transferred group (Figure 4.20 B) compared to both Th17 groups. Significantly more Th1 donor cells were found in the CNS compared to the very low numbers of both groups of Th17 donor cells (Figure 4.20 C). In summary, despite the increase in PSGL-1 expression on the 'Th17+IL-12' cells this did not allow them access to the non-inflamed CNS to induce disease.

4.3 Discussion

T cell subsets differentially express selectins, adhesion molecules and chemokine receptors thereby suggesting a differential ability to enter both non-inflamed and inflamed sites.

As shown in Chapter 3, *in vitro* polarised Th1 cells were able to infiltrate into the non-inflamed CNS whereas *in vitro* polarised Th17 cells were only able to accumulate in the CNS once inflammation was already established. Development of a therapy that specifically blocks the initial migration of Th1 cells into the CNS should prevent the initiation of inflammation within the CNS. The use of an inhibitor that specifically targets the pro-inflammatory T cell subset causing the inflammation would be an attractive therapy as it would result in minimising side effects.

In vitro polarised Th1 cells had a higher expression of CD62L compared to *in vitro* polarised Th17 cells. CD62L (or L-selectin) is known to be expressed on naïve T cells and not on memory T cells, although these can then re-express CD62L to aid their homing to particular sites highlighting that CD62L follows a cyclical pattern of expression (Klinger et al., 2009). A carbohydrate binding protein, CD62L is required for the entry of naïve T cells into the peripheral lymph nodes via adhesion to the high endothelial venules of the peripheral lymph nodes (Geoffroy and Rosen, 1989; Bradley, Watson and Swain, 1994). This process allows for immune surveillance to occur and for the accumulation of antigen specific lymphocytes at the site of antigen exposure. Conflicting reports using different EAE models make it unclear as to whether CD62L is important for the induction or the effector phase of EAE. It has been shown that *in vivo* treatment with HRL3, another monoclonal antibody against CD62L, led to inhibition of active EAE in Lewis rats, and had a mild inhibitory effect on adoptive transfer (Archelos et al., 1998). Fewer inflammatory infiltrates were found in the CNS as well as a depletion of T cells both in the lymph nodes and the spleen (Archelos et al., 1998). Interestingly it has also been suggested that CD62L is required for the actual damage to occur to the myelin within the CNS. This was shown by CD62L deficient MBP-TCR transgenic mice being resistant to disease

despite a large infiltration of T cells being observed in the CNS. The resistance to disease was due to an absence of myelin damage (Grewal et al., 2001) within the CNS. In contrast Li and colleagues have suggested CD62L is required for the induction phase of EAE by showing that CD62L-deficient mice were resistant to MOG-induced EAE due to lack of priming of the T cells in the periphery (Li et al., 2006).

Contrary to this, with the use of another transgenic model, it was found that passive transfer of MBP (Ac1-11) specific T cells into (PL x SJL)F_1 mice and subsequent treatment with Mel-14 had no effect on EAE induction compared to the PBS controls (Brocke et al., 1999). Supporting the case for CD62L not being required for EAE, it has recently been observed that CD62L-deficient SJL and C57BL/6 mice are both fully susceptible to PLP-induced and MOG-induced EAE respectively (Uboldi et al., 2008).

Here, the treatment of the host mice with Mel-14 led to a significant decrease in the number of Tg4 Th1 donor cells found in the inguinal lymph nodes, correlating well with the requirement of CD62L for entry of T cells into peripheral lymph nodes, and confirming that the antibody was indeed having an effect *in vivo*. However, CD62L expression seemed not to be required for the entry of Tg4 Th1 donor cells into the CNS as these were still readily able to migrate to the CNS after Mel-14 treatment. The discrepancies in contradictory results regarding the role of CD62L in EAE induction could be reflective of the different genetic backgrounds of the various polyclonal/transgenic mice used in the models.

Higher levels of soluble CD62L have been found in the serum of MS patients compared to healthy controls (Mossner et al., 1996; Duran et al., 1999). The level of soluble CD62L in the serum correlated with the size of enhancing lesions in the CNS, as well as with the levels of soluble CD62L in the CSF (Mossner et al., 1996) (the level of soluble CD62L being reflective of the amount of CD62L shed to enter a site of inflammation) suggesting CD62L may indeed have a role in MS pathogenesis.

Previously, when MS and EAE were still considered to be Th1 mediated autoimmune diseases, it was shown that human Th1 clones express CCR5 and CXCR3 (Bonecchi et al., 1998; Sallusto et al., 1998). $CCR5^+$ and $CXCR3^+$ T cells have been found to be increased in numbers in the blood and CSF of MS patients (Teleshova et al 2002) (Balashov et al., 1999; Teleshova et al., 2002). In addition, in the progressive form of MS, increased numbers of $CCR5^+$ and $CXCR3^+$ T cells were found in peripheral blood compared to healthy controls. The ligands of CCR5 and CXCR3 were also found associated with microglia/macrophages and astrocytes in MS lesions (Balashov et al., 1999).

The *in vitro* polarised Th1 cells used here expressed CCR5 to a higher level than their Th17 counterparts. CCR5 expression is upregulated in the presence of IL-12 (Bagaeva, Williams and Segal, 2003), included in the Th1-polarising cocktail used here. Surprisingly, although CXCR3 was raised on Th1 cells here, this was not a specific feature of the Th1 cells as it was also expressed on Th17 cells to the same level. For this reason, the blockade of CXCR3 expression was not actively pursued here. It is interesting to note that the *in vitro* polarised Th1 and Th17 cells appeared to express the same protein level of CXCR3 and CCR6, two chemokine receptors normally associated with Th1 or Th17 cells respectively.

Tak779 is a small, non-peptide antagonist of CCR5, CXCR3 and, to a lesser extent, CCR2 (Baba et al., 1999; Gao et al., 2003). Tak779 antagonises the binding of the CCR5 ligand, RANTES to $CCR5^+$ cells and thereby blocks CCR5-mediated $Ca2^+$ signalling (Baba et al., 1999; Gao et al., 2003). The lack of efficacy of Tak779 seen here suggests that CCR5 expression on the Tg4 Th1 cells was not required for the entry of the donor Th1 cells into the CNS to induce disease. This is consistent with some previous studies. For example, C57BL/6 mice deficient in CCR5 or one of its ligands, MIP-1α, are both still susceptible to MOG-induced EAE (Tran, Kuziel and Owens, 2000).

However, efficacy of the dose of Tak779 used here in the Tg4 passive transfer system, or its stability *in vivo* was not demonstrated. The dose used, 50 μg

(equivalent to 2 mg/kg), was chosen as this dose was shown to have an effect in a model of adjuvant arthritis in Lewis rats (Okamoto and Kamatani, 2006). Here, due to using the intravenous route of injection, the frequency of administration was reduced to every two days, rather than everyday. The lack of effect observed here could be due to the low efficacy of Tak779 and a short half-life of the antagonist *in vivo*. Unfortunately, due to the absence of a positive control showing the efficacy of Tak779, it cannot be definitively concluded from this data that Tak779 had no effect on entry of the donor cells into the CNS. In addition, the use of a 'scrambled peptide' control of Tak779 would have been a more appropriate negative control as opposed to PBS.

The *in vitro* pre-treatment of Th1 cells with an antibody against CCR5 prior to transfer also had no affect on EAE induction (Richard O'Connor, personal communication). In contrast, in experimental autoimmune uveitis (EAU) pre-treating donor T cells with an antibody against CCR5 led to a reduction of their infiltration into the retina (Crane et al., 2006). However, these cannot be directly compared as in EAU, donor cells can enter the retina within hours of transfer, whereas it takes days in EAE, by which time the antibody could have worn off the donor T cells *in vivo*.

Recently MOG-induced active EAE in C57BL/6 mice was reported to be reduced both in severity and incidence by the treatment of Tak779 everyday by subcutaneous injection (Ni et al., 2009). In correlation with data shown here, Ni and colleagues also showed that Tak779 had no effect on the transfer of EAE by passive transfer of MOG-specific encephalitogenic T cells highlighting differences between the active and passive induction of EAE.

Surprisingly, *in vitro* polarised Th1 cells exhibited surface CCR6 expression, a chemokine receptor normally associated with Th17 cells. CCR6 has been shown to be upregulated in the spinal cord during EAE (Liston et al., 2009) and is thought to be important in Th17 mediated disease (Reboldi et al., 2009). It is considered essential for the entry of encephalitogenic T cells into the CNS via the choroid plexus in particular (Reboldi et al., 2009). However, CCR6 surface expression was

also observed on Th1 cells here. In contrast, when the mRNA expression of CCR6 was determined, Th1 cells were found to have very low levels, and CCR6 was clearly highly upregulated on the Th17 cells. Explanations for this could be that the Th1 cells exhibit instability of the CCR6 mRNA, and/or the protein half-life is extended in the Th1 cells, and/or the translation of the CCR6 protein is more efficient in the Th1 cells, as the flow cytometry analysis clearly showed strong positive staining of CCR6. Despite this, surface expression of CCR6 on both the Th1 and Th17 cells does not explain how, as shown in this thesis, the Th1 cells are able to enter the CNS and induce disease and not the Th17 cells.

PSGL-1 is required for the initial tethering of the lymphocyte to the endothelial wall. Increased expression of PSGL-1 has been found on cells from human CSF compared to the peripheral blood, and expression of P-selectin was also found to be increased, specifically in the vasculature of the choroid plexus, suggesting a role for this PSGL-1/P-selectin interaction in migration of T cells into the CNS (Kivisakk et al., 2003). Here, PSGL-1 was expressed to a higher level on *in vitro* polarised Th1 cells than Th17 cells.

In general, PSGL-1 is expressed on the surface of most circulating myeloid cells, dendritic cells and T lymphocytes (as well as other lymphoid cells) (Laszik et al., 1996). Despite the surface expression of PSGL-1 on a broad spectrum of cells, its surface expression does not necessarily correlate to its functional ability. It has been shown that Th1 and Th2 cells express PSGL-1 to the same extent, however only Th1 cells are able to bind to P-selectin via PSGL-1; whereas Th2 cells cannot (Borges et al., 1997). Functional PSGL-1 expression on Th1 cells is required for their entry into the inflamed skin via binding to P-selectin (Austrup et al., 1997; Borges et al., 1997; Tietz et al., 1998). Similarly, it has been shown that PSGL-1 is required for Th1 cells to cross the blood-retina barrier in EAU (Xu et al., 2004). Again, this was not a function of Th2 cells, and pre-treatment of cells with an anti-PSGL-1 antibody inhibited rolling and infiltration of Th1 cells specifically into the eye (Xu et al., 2004). The mechanism, by which PSGL-1 functions to aid T cell arrest and migration across the endothelial wall, is thought to be mediated through enhancing of LFA-1

dependent cell binding to ICAM-1. This increased binding leads to integrin activation and Th1 cell arrest on the surface of the vascular endothelium and subsequent migration into the target tissue (Atarashi et al., 2005). Here, it was investigated whether, in the same way as it is required for entry into the skin, and the eye in EAU, PSGL-1 is required on *in vitro* polarised Th1 cells to enter the CNS and induce EAE.

Pre-treatment with anti-PSGL-1 antibody (4RA10) led to a delay in disease onset compared to the PBS treated controls. Previous work with this blocking antibody had shown that using the antibody in the same way (pre-treating cells prior to transfer) led to the induction of milder EAE (Deshpande, King and Segal, 2006). Although a lower severity of EAE was not seen here, the delay in onset correlates with this observation. A trend towards fewer donor Th1 cells being able to infiltrate across the BBB and into the CNS at a pre-clinical time point, suggests PSGL-1 is required at a very early stage as pathogenic T cells populate the CNS. However, the kinetics of the effects of the blocking antibody could mean that the effects of the antibody wear off *in vivo* after a few days, leading to the donor Tg4 Th1 cells being able to later enter the CNS and induce a normal level of disease.

To overcome this potential difficulty, the *in vivo* administration of the blocking antibody was used to determine if this would abrogate EAE induction completely. Initial results were promising and showed that pre-treating the donor Tg4 Th1 cells with the blocking antibody prior to transfer, followed by *in vivo* administration of 25 μg of 4RA10 by intravenous injection every two days from day 1 post-transfer, led to the inhibition of disease induction for the duration of treatment. The last dose of antibody was given on day 11 post-transfer, and one out of the three host mice developed a mild level (maximum score of 2) of EAE on day 13 post-transfer, two days after the last administration of blocking antibody. This was a strong indication of the need of functional PSGL-1 expression on Tg4 Th1 cells for the infiltration into the CNS and subsequent induction of inflammation and EAE.

On repeat, the host mice were culled on day 7 post-transfer due to apparent anaphylaxis of some of the mice after antibody treatment. Despite fewer total cells being present in the CNS after *in vivo* treatment with the blocking antibody, this lower number of cells did not correlate to fewer donor Th1 cells entering the CNS. In the same way, there was no significant difference in the percentage or number of $CD11b^+$ cells in the CNS at this time-point, indicating the anti-PSGL-1 treatment had no effect on the number of donor Th1 or $CD11b^+$ cells in the CNS. In contrast, significantly lower frequencies and absolute numbers of $CD11b^+$ cells were found in the spleen suggesting the treatment reduced the number of $CD11b^+$ cells in the spleen. This unexpected result correlates with the expression of PSGL-1 on monocytes and macrophages (An et al., 2008) suggesting PSGL-1 may be required for the migration of macrophages to the spleen. Importantly, the key difference between the experiment presented previously and this was the presence/absence of the *in vitro* blocking step, prior to transfer of the cells. Therefore, the initial blocking of PSGL-1 function prior to transfer may confer the important window of time necessary to inhibit the induction of EAE via the inhibition of migration of donor Tg4 Th1 cells into the CNS. The use of PBS as the control for the initial *in vivo* experiment is a limitation on the results. The repeat experiment using the IgG1 isotype control where no difference was observed in terms of migration, could indeed reflect the true result. A repeat of this experiment, again with the appropriate IgG1 isotype control, would give a definitive conclusion to these sets of experiments.

The literature available on the role of PSGL-1 in EAE induction is contradictory. PSGL-1-deficient C57BL/6 mice were susceptible to the induction of MOG-induced active EAE (Engelhardt et al., 2005; Osmers, Bullard and Barnum, 2005). In the same respect, SJL mice did not have an increased expression of E- or P-selectin during active PLP-induced EAE, suggesting that these molecules are not required for entry of T cells into the CNS (Engelhardt et al., 1997). SJL mice were also susceptible to PLP-induced active EAE when treated with 4RA10 anti-PSGL-1 antibody (Engelhardt et al., 2005).

In contrast, blocking PSGL-1 by pre-treating donor cells with 4RA10 led to the development of milder EAE after transfer to B10.PL hosts (Deshpande, King and Segal, 2006). This is consistent with the delayed course of EAE shown here, correlating with fewer donor cells entering the CNS. The anomalies seen between the different models could reflect the different genetic backgrounds of the mouse strains used here i.e. differences between the polyclonal C57BL/6 and SJL mice, compared to the transgenic system using transgenic T cells for passive transfer into B10.PL (or B10.PLxC57BL/6) host mice.

Data here would suggest that pre-treatment of Tg4 Th1 T cells with an anti-PSGL-1 antibody does indeed impair the entry of the donor cells into the CNS across the BBB. Pre-treating the donor Tg4 Th1 cells and subsequently blocking PSGL-1 *in vivo*, led to the inhibition of EAE for the duration of treatment, suggesting PSGL-1 signalling is required for the induction of EAE. However, increasing the surface expression of PSGL-1 on *in vitro* polarised Th17 cells did not confer their pathogenicity *in vivo*. This of course could be due to the increased expression of PSGL-1 seen on the 'Th17+IL-12' cells not being functional. The expression of the two enzymes C2GnT-I and FucT-VII on the 'Th17+IL-12' cells needs to be determined to deduce whether the PSGL-1 seen here is indeed functional.

4.4 Conclusions

- *In vitro* polarised Th1 cells express higher levels of CCR5, CD62L and PSGL-1 compared to Th17 cells.

- Of these, only blockade of PSGL-1 could affect the clinical course of EAE by passive transfer of Tg4 Th1 cells, leading to a delay in EAE induction. This correlated with their impaired entry to the CNS at a pre-clinical time-point.

- Pre-treatment of Tg4 Th1 cells with an anti-PSGL-1 blocking antibody, and subsequent *in vivo* blockade of PSGL-1, completely abrogated EAE for the duration of treatment.

- Increasing the surface expression of PSGL-1 (and CCR5) on Th17 cells through IL-12 did not result in the Th17 cells gaining pathogenic abilities.

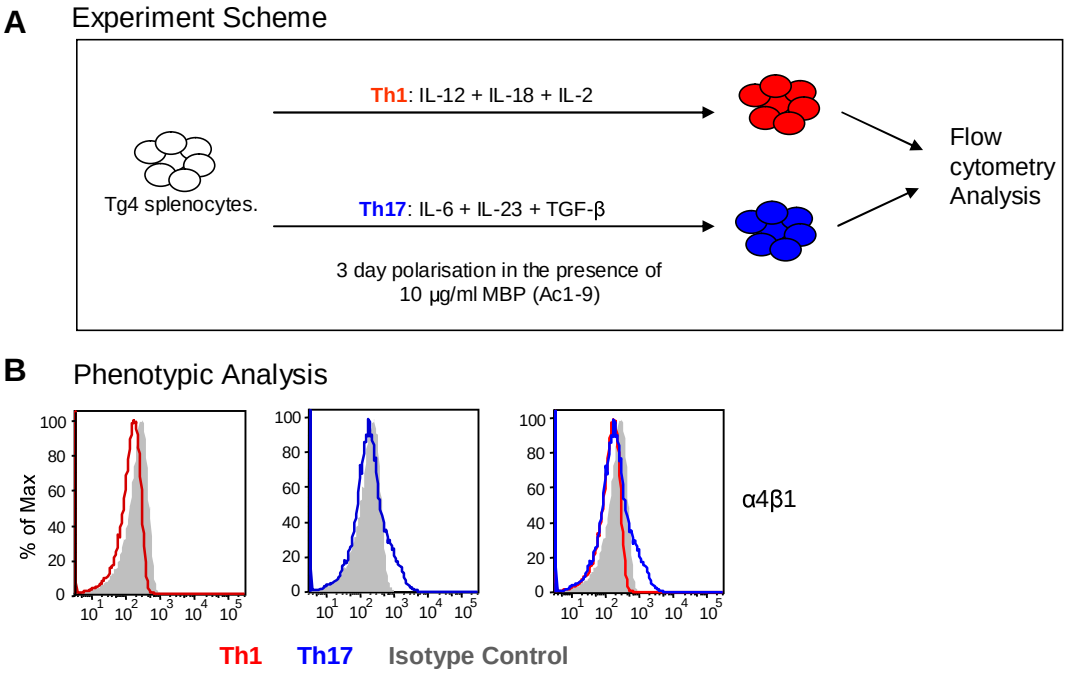

Figure 4.1 *In vitro* polarised Tg4 Th1 cells do not express higher levels of α4β1-integrin (VLA-4) than Tg4 Th17 cells

A, Experimental scheme; **B,** Gated on $CD4^+$ cells, α4β1-integrin expression on (left) Tg4 Th1 (**red**) and (middle) Th17 (**blue**) cells versus the isotype control (**grey fill**) and (right) overlay. Representative of two experiments showing the same result.

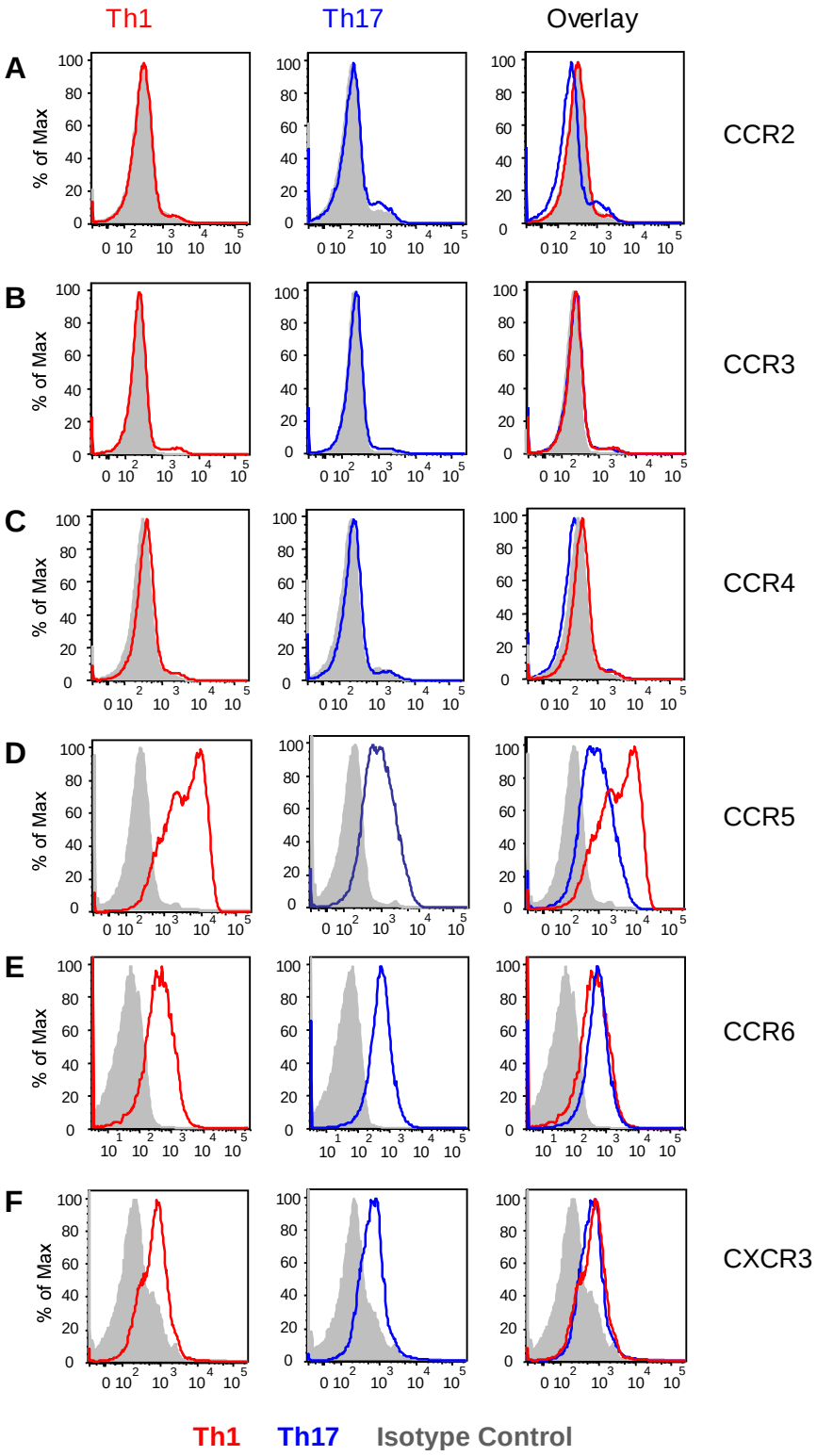

Figure 4.2 Expression of chemokine receptors on Tg4 Th1 and Th17 *in vitro* polarised cells.

Gated on $CD4^+$ cells, expression of A, CCR2, B, CCR3, C, CCR4, D, CCR5, E, CCR6 and F, CXCR3 on *in vitro* polarised (left) Tg4 Th1 (**red**), (middle) Th17 (**blue**) cells versus the appropriate isotype controls (**grey fill**) and (right) overlay. Representative of three experiments.

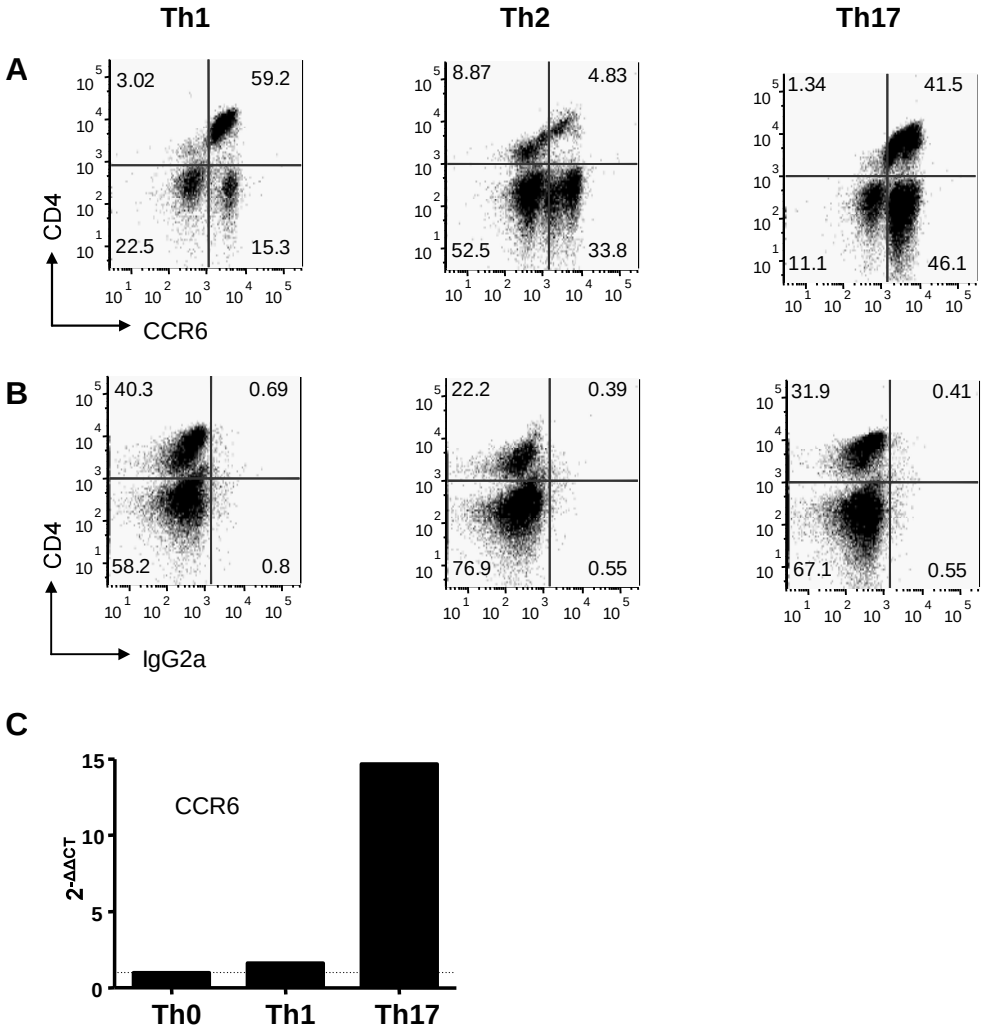

Figure 4.3 *In vitro* polarised Th1 and Th17 cells both express chemokine receptor CCR6 as determined by flow cytometry and RT-qPCR .

A, Gated on live lymphocytes, CCR6 expression on $CD4^+$ cells in Th1 (left), Th2 (middle) and Th17 (right) *in vitro* polarised cells (anti-CD3 stimulated C57BL/6 splenocytes). **B,** Gated on live lymphocytes, isotype control IgG2a versus CD4; Th2 cells were polarised by Dominika Nowakowska. **C,** mRNA expression level of CCR6 as compared to Th0 control cells (cells stimulated in the absence of exogenous cytokines). $2^{-\Delta\Delta CT}$ = 1 for the control sample. Expression above this level indicates upregulation. RT-qPCR representative of 2 separate experiments. Flow representative of two experiments for the Th1/Th17 cells and one experiment for the Th2 CCR6 expression.

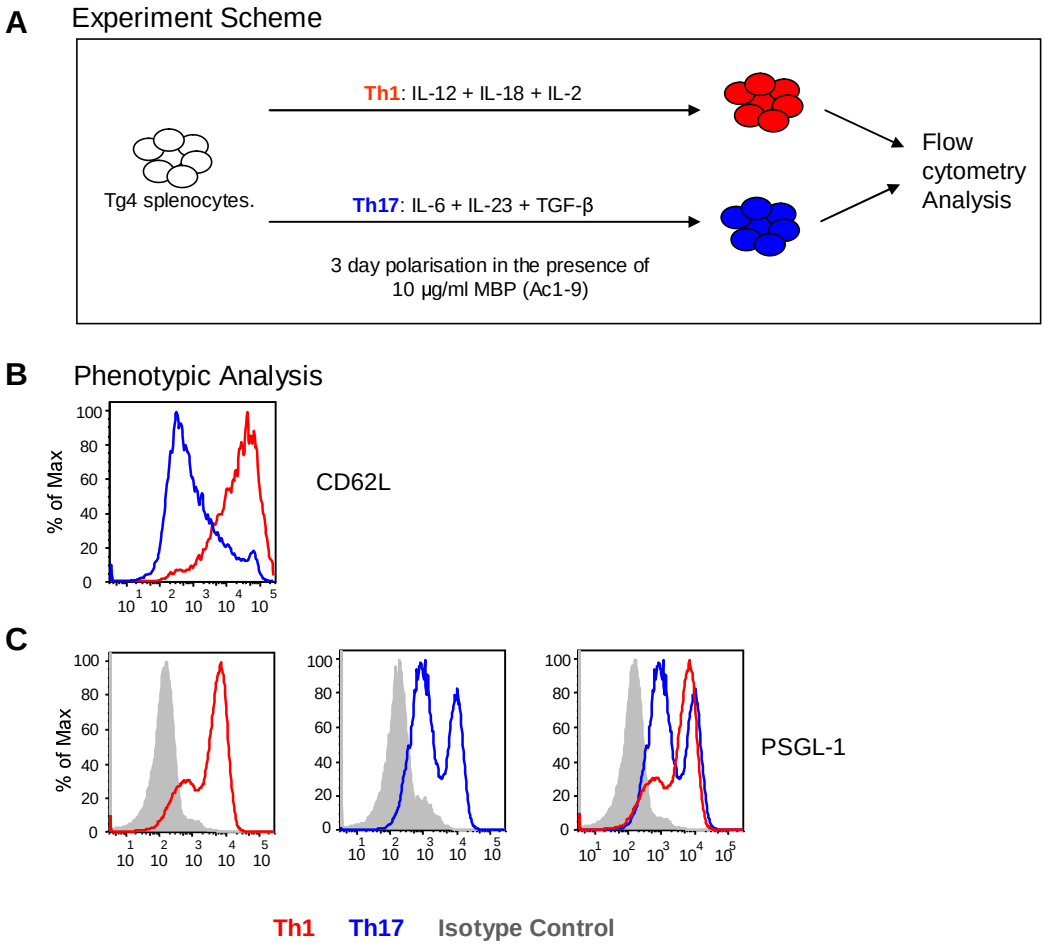

Figure 4.4 Tg4 Th1 *in vitro* polarised cells express higher levels of CD62L and PSGL-1 compared to Th17 cells.

A, Experimental scheme; **B,** Gated on $CD4^+$ cells, CD62L expression on Tg4 Th1 (**red**) and Th17 (**blue**) cells; **C,** Gated on $CD4^+$ cells, PSGL-1 expression on Tg4 Th1 (**red**), Th17 (**blue**) and IgG1 isotype control (**grey fill**). See Appendix 7 for flow cytometry gating strategy for CD62L, representative of Tg4 Th1 and Th17 phenotypic analysis. Expression analysed for most Tg4 passive transfer experiments performed.

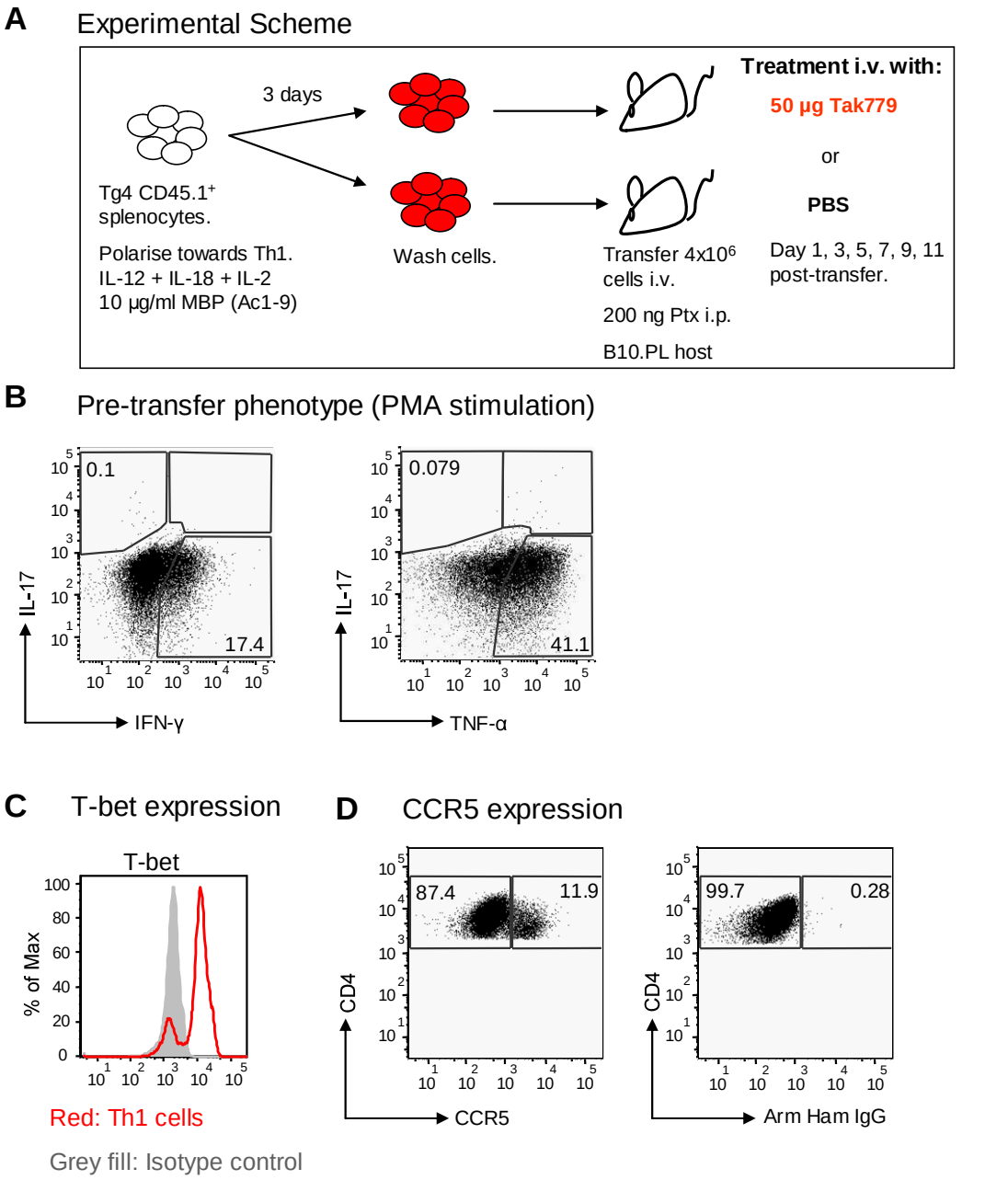

Figure 4.5 Effect of *in vivo* treatment with CCR5 inhibitor Tak779 on EAE induction and development.

A, Experiment scheme; Pre-transfer phenotype of Tg4 Th1 cells showing **B,** IFN-γ, TNF-α and IL-17 production, gated on the $CD4^+$ cells; **C,** T-bet expression gated on $CD4^+$ cells showing T-bet (**red**) and IgG1 isotype control (**grey fill**); **D,** CCR5 expression gated on $CD4^+$ cells and the isotype control. Representative of two experiments.

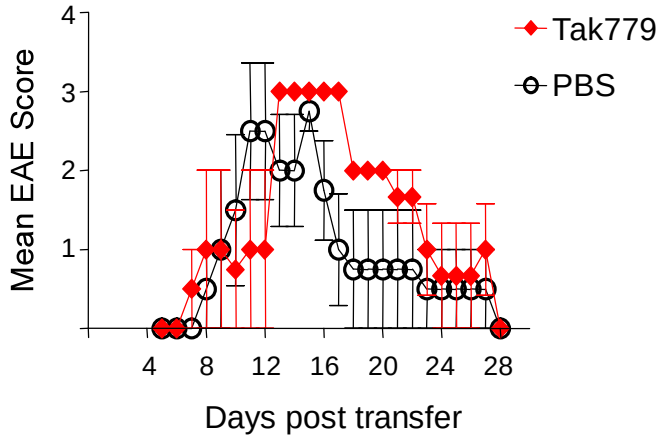

Figure 4.6 Treatment with Tak779 has no effect on EAE induction by adoptive transfer of Tg4 Th1 cells.

Mean EAE scores versus days post transfer of mice treated with PBS (○) or 50 µg Tak779 (♦) i.v. on days 1, 3, 5, 7, 9, 11 post-transfer. Disease incidence: PBS (4/4), Tak779 (3/4). Results are representative of two experiments. No significant difference in EAE severity between PBS and Tak779 treated groups, as determined by Fisher's exact test (p=1.0000). Error bars represent mean ± standard error. Representative of two experiments.

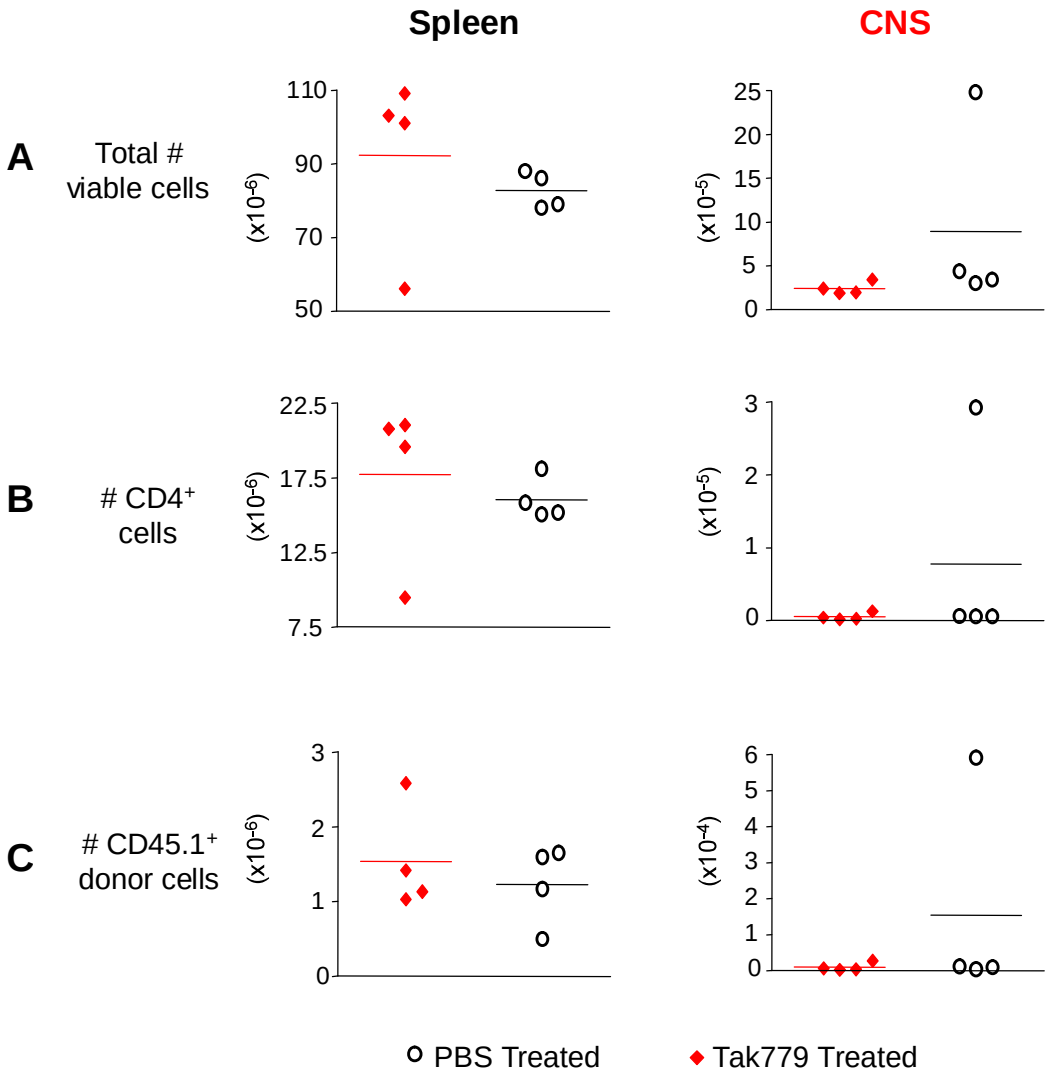

Figure 4.7 Location of Tg4 Th1 CD45.1 donor cells in the periphery and CNS after *in vivo* treatment with Tak779.

A, Total number of viable cells, **B,** total number $CD4^+$ cells and **C,** total number of Tg4 Th1 $CD45.1^+$ donor cells entering the spleen (left) and CNS (right) at day 7 post-transfer for PBS treated (○) or Tak779 treated (♦) mice. Statistics performed with Mann Whitney Test. See Appendix 8 for representative flow cytometry gating strategy for presence of donor T cells. n=4 for both groups. Representative of two experiments.

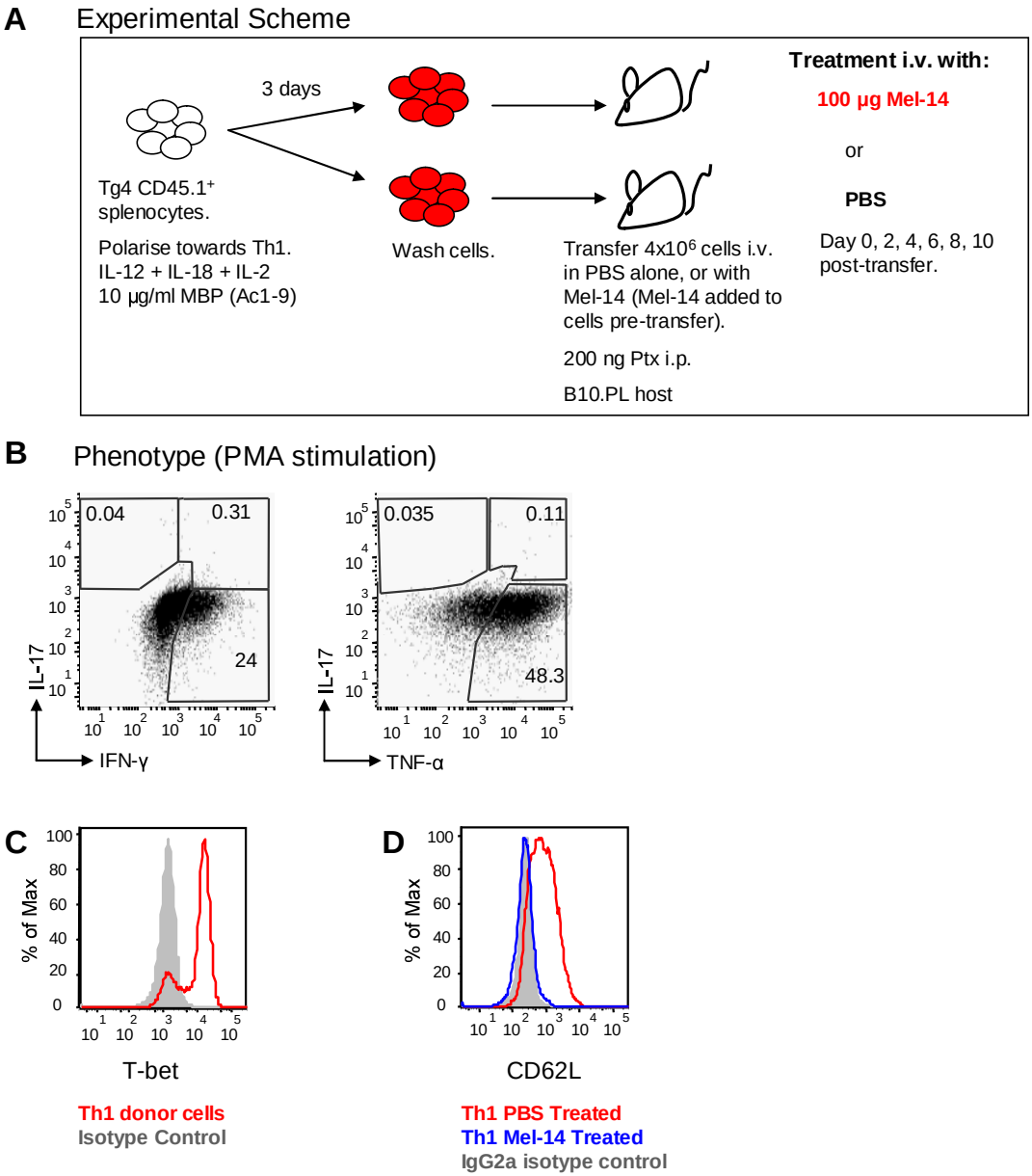

Figure 4.8 Pre-transfer phenotype of Tg4 Th1 cells prior to treatment with Mel-14 *in vivo*.

A, Experiment scheme; Phenotype of Tg4 Th1 polarised cells after PMA stimulation, gated on $CD4^+$ cells showing **B**, IFN-γ, IL-17 and TNF-α production; **C,** T-bet expression on Tg4 Th1 cells (**red**) versus IgG1 isotype control (**grey fill**) gated on $CD4^+$ cells; **D,** CD62L expression on PBS treated Tg4 Th1 cells (**red**) versus Mel-14 treated Tg4 Th1 cells (**blue**) and IgG2a isotype control (**grey fill**). Representative of two experiments.

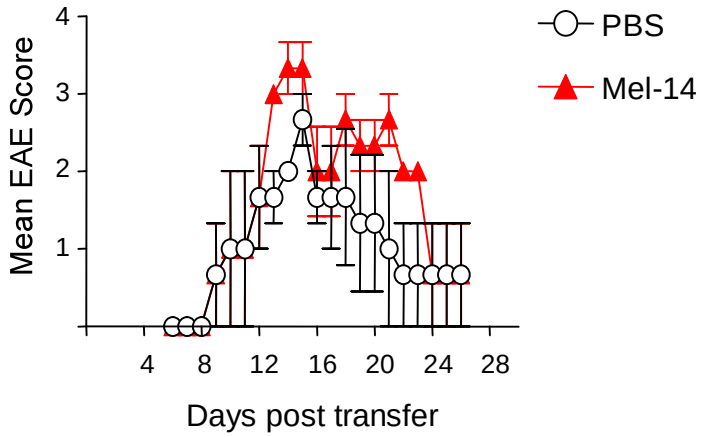

Figure 4.9 Treatment with Mel-14 has no effect on EAE induction or development.

Mean EAE score versus days post cell transfer for Mel-14 treated (▲) versus PBS treated mice (○). Mice treated on days 0, 2, 4, 6, 8, 10 post-transfer with 100 μg Mel-14 or PBS i.v. Disease incidence: PBS (3/3); Mel-14 (3/3). Results representative of two experiments. No significant difference in EAE severity between the PBS treated and Mel-14 treated groups, as determined by Fisher's exact test ($p=1.0000$). Error bars represent mean ± standard error.

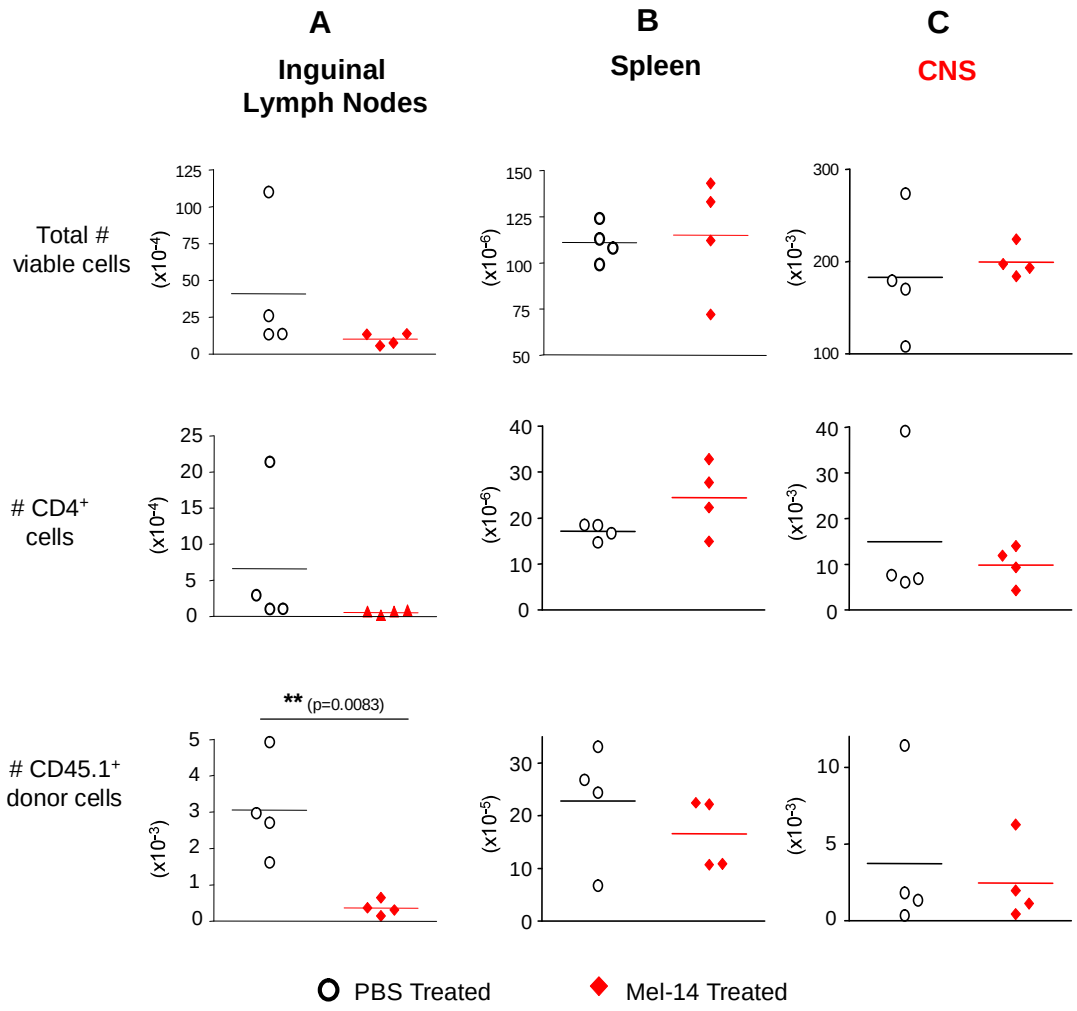

Figure 4.10 Treatment of mice with Mel-14 has no effect on the migration of Tg4 Th1 donor cells in to the CNS.

Total number of viable cells, total $CD4^+$ cells and absolute number of $CD45.1^+$ Tg4 Th1 donor cells entering the **A,** inguinal lymph nodes, **B,** spleen and **C,** CNS at day 5 post-transfer (pre-clinical) for PBS treated (○) and Mel-14 treated (♦). Results are representative of two experiments. n=4 for both groups. Statistics performed using Mann Whitney Test.

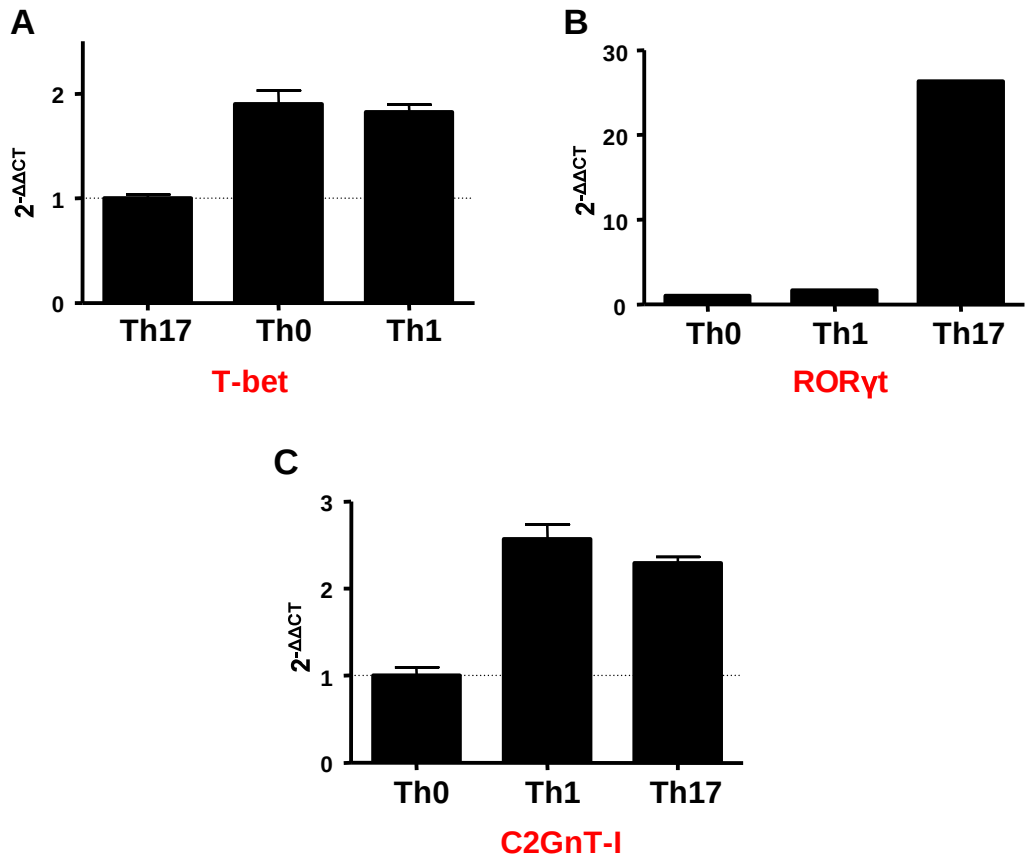

Figure 4.11 Expression of C2GnT-I on *in vitro* polarised Th1 and Th17 cells.

In vitro polarised Tg4 Th0, Th1 and Th17 cells showing mRNA expression of **A,** T-bet, **B,** RORγt and **C,** C2GnT-I, as determined by RT-qPCR. HPRT used as the control house-keeping gene. T-bet expression calculated using Th17 as the control samples; RORγt expression calculated using Th0 as the control sample; C2GnT-I expression calculated using Th0 as the control sample. Error bars represent standard deviation in replicate samples. Dotted line denote $2^{-\Delta\Delta CT}$ = 1 of control sample. Representative of two experiments.

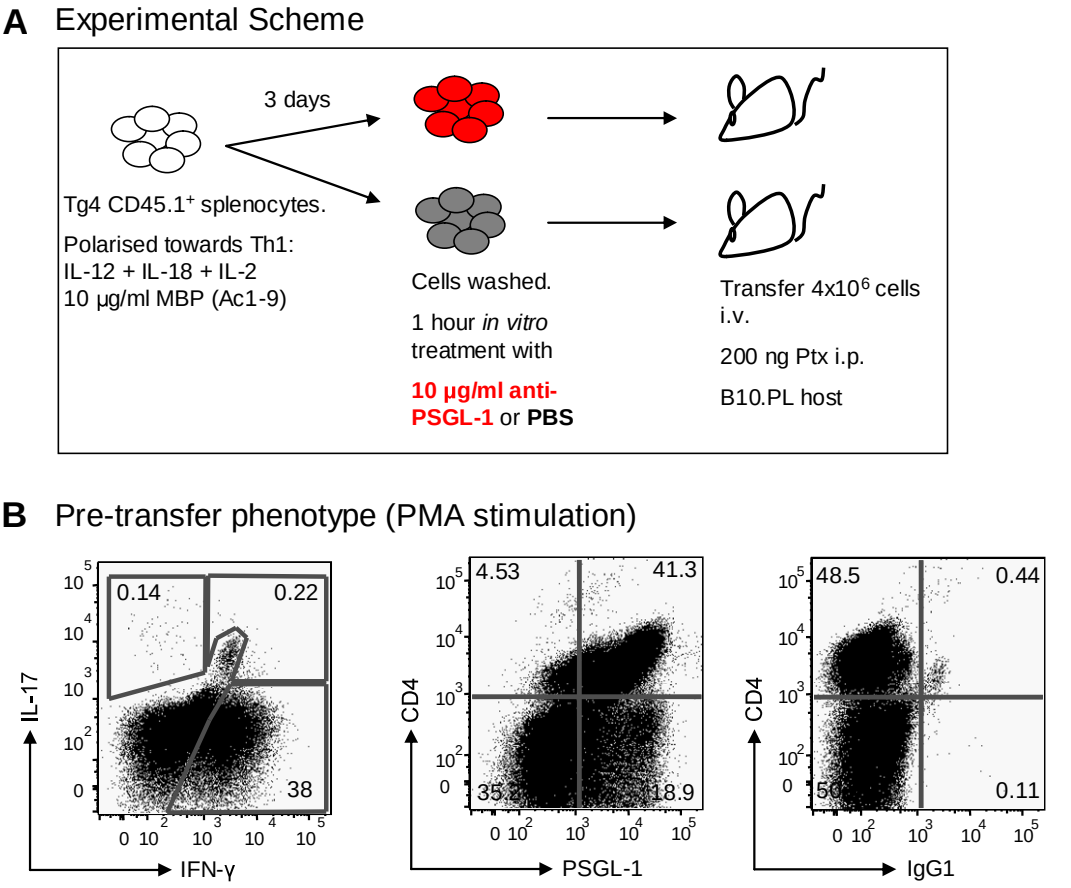

Figure 4.12 Tg4 Th1 cells treated with anti-PSGL-1 prior to transfer induce a delayed course of disease.

A, Experiment scheme; **B,** Pre-transfer phenotype of Tg4 Th1 polarised cells showing IFN-γ, IL-17 and TNF-α production, gated on $CD4^+$ T cells, and PSGL-1 surface expression gated on live lymphocytes. Staining representative of numerous Tg4 Th1 passive transfer experiments with pre-transfer phenotyping of Tg1 Th1 cells, another example of which is shown in Figure 4.15.

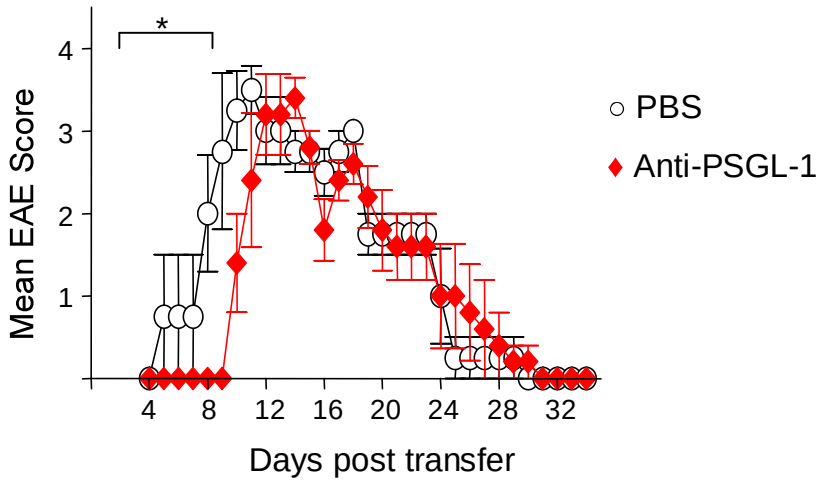

Figure 4.13 Pre-treatment of Tg4 Th1 cells with anti-PSGL-1 resulted in a delay in disease induction.

In vitro polarised Tg4 Th1 cells were pre-treated with 10 μg/ml anti-PSGL-1 or PBS for one hour prior to transfer. Mean clinical EAE scores versus days post-transfer. Disease incidence: PBS (4/4), anti-PSGL-1 (5/5). There is a significant delay in the induction of disease in the anti-PSGL-1 pre-treated group compared to the PBS pre-treated group, as determined by Mann Whitney test (* p=0.0317). Error bars represent mean ± standard error. Representative of two experiments, see Appendix 9 for EAE scores for second experiment.

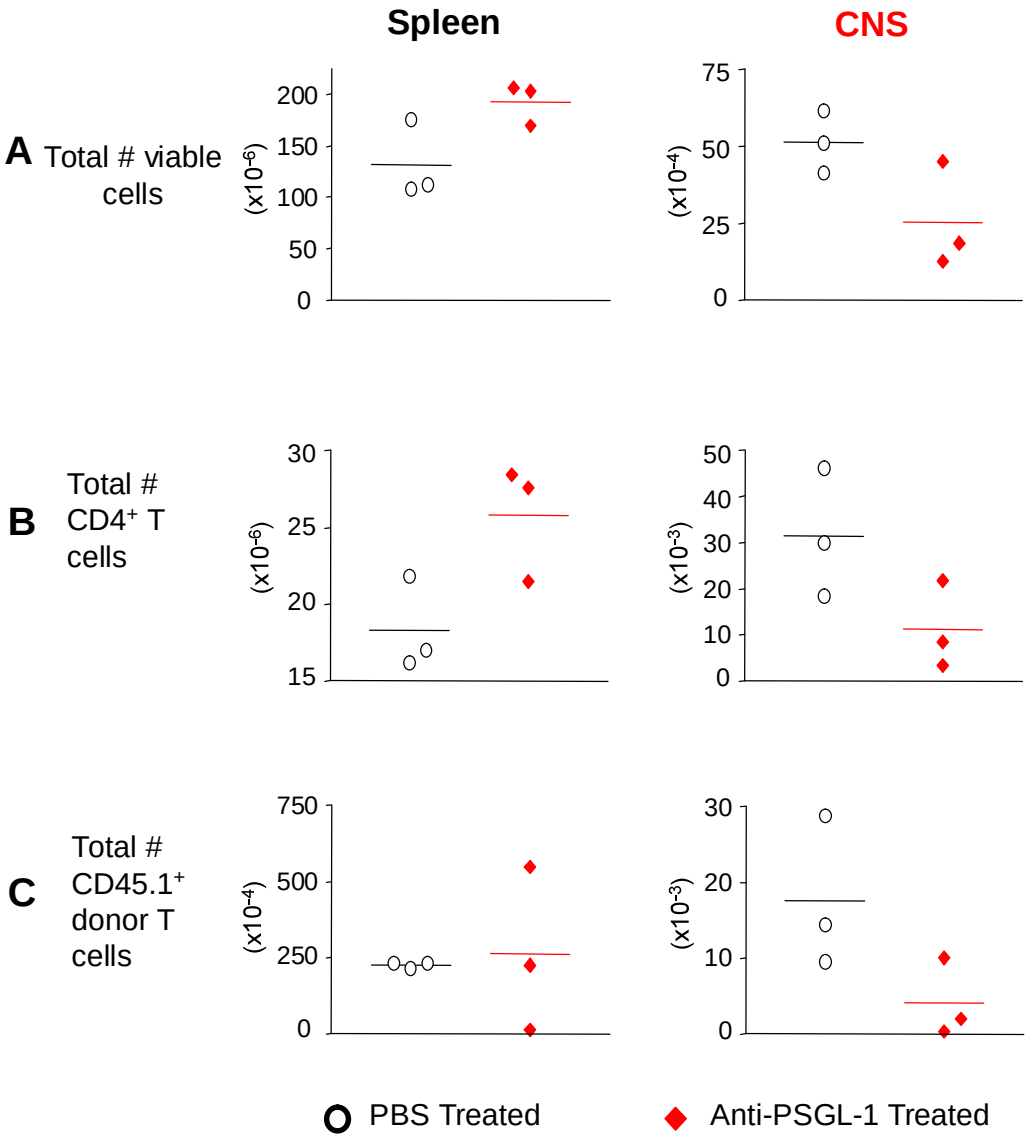

Figure 4.14 Anti-PSGL-1 pre-treatment resulted in a trend towards fewer Tg4 Th1 donor cells entering the CNS at a pre-clinical stage.

A, Total number of viable cells; **B,** Total number of $CD4^+$ T cells; **C,** Total number of $CD45.1^+$ donor Th1 cells in spleen (left) and CNS (right) taken at day 4 post-transfer, PBS (○) versus anti-PSGL-1 (♦) *in vitro* treated Th1 cells; Statistics performed using a Mann Whitney test. n=3 for both groups. Pre-clinical harvest representative of four experiments.

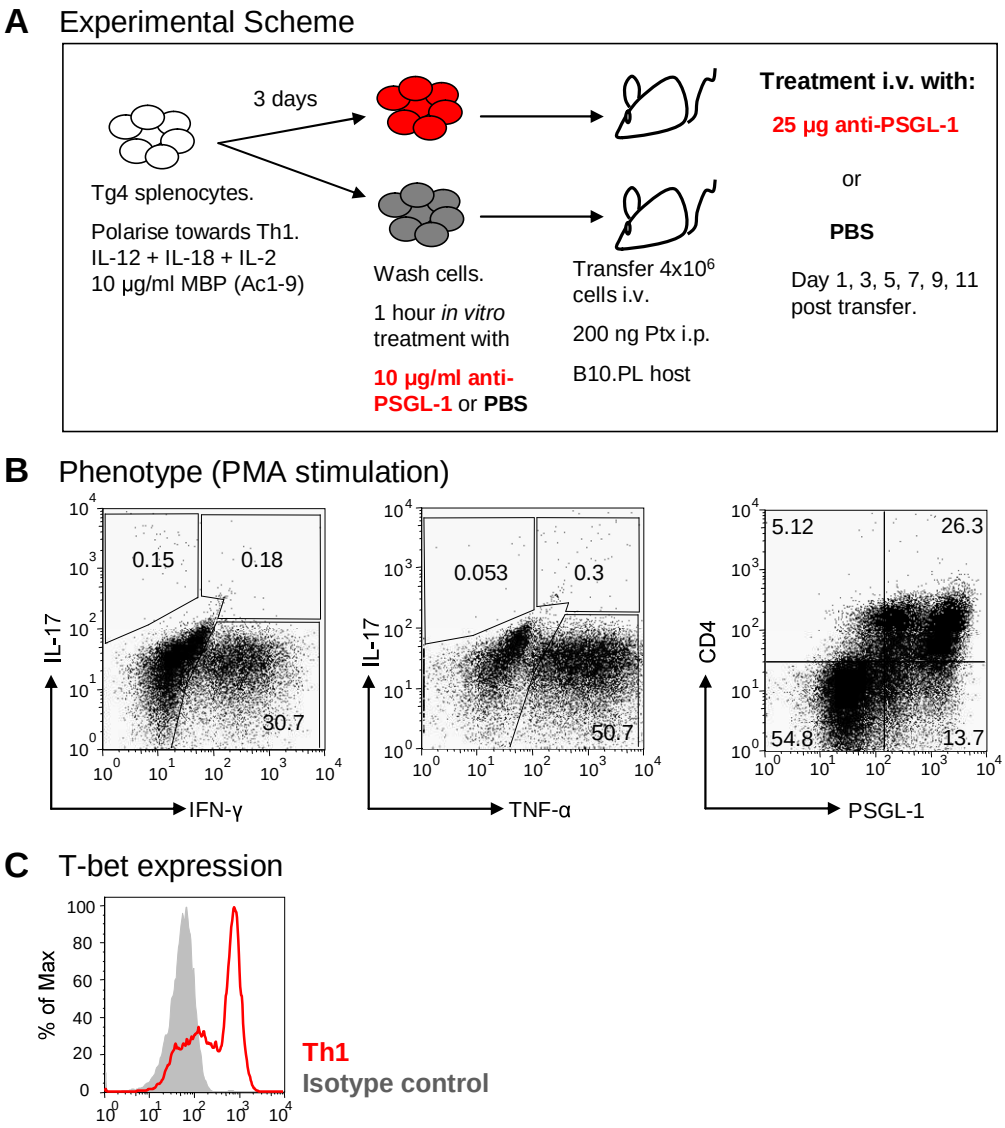

Figure 4.15 Effect of *in vivo* treatment of anti-PSGL-1 on Tg4 Th1 passive transfer EAE.

A, Experiment scheme; **B,** pre-transfer phenotype of Tg4 Th1 cells showing IFN-γ, TNF-α and IL-17 production, gated on $CD4^+$ cells, and PSGL-1 expression gated on live lymphocytes; **C,** T-bet expression on Tg4 Th1 cells, gated on $CD4^+$ cells showing T-bet (**red**) versus IgG1 isotype control (**grey fill**). Staining is representative of numerous Tg4 Th1 passive transfer experiments with pre-transfer phenotyping, another example of which is shown in Figure 4.12.

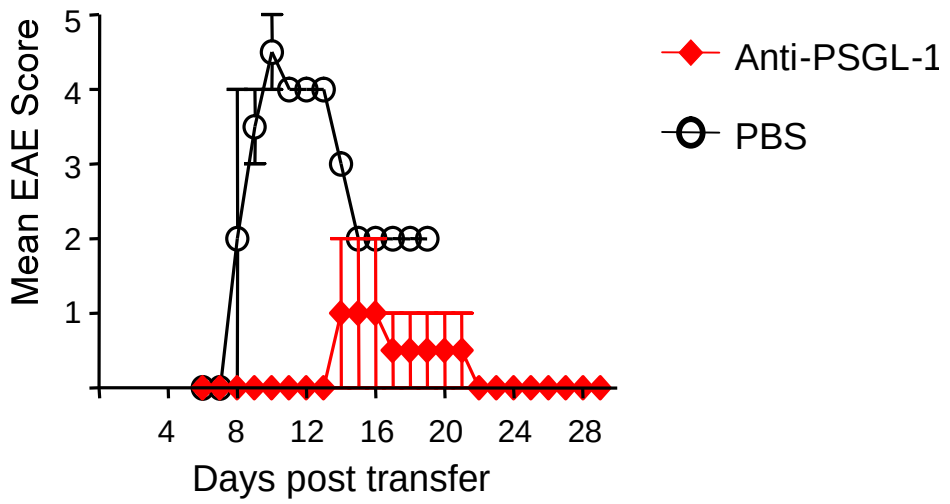

Figure 4.16 Effect of *in vivo* treatment of anti-PSGL-1 on Tg4 Th1 passive transfer EAE.

Mean EAE scores versus days post-transfer of PBS (○) and anti-PSGL-1 (♦) treated mice. Host mice treated with anti-PSGL-1 or PBS i.v. on days 1, 3, 5, 7, 9, 11 post-transfer. Disease incidence: PBS (2/2), anti-PSGL-1 (1/3). Error bars represent mean ± standard error. No significant difference in EAE severity between PBS or anti-PSGL-1 *in vivo* treated groups, as determined by Fisher's exact test (p=0.1000). EAE scores representative of one experiment.

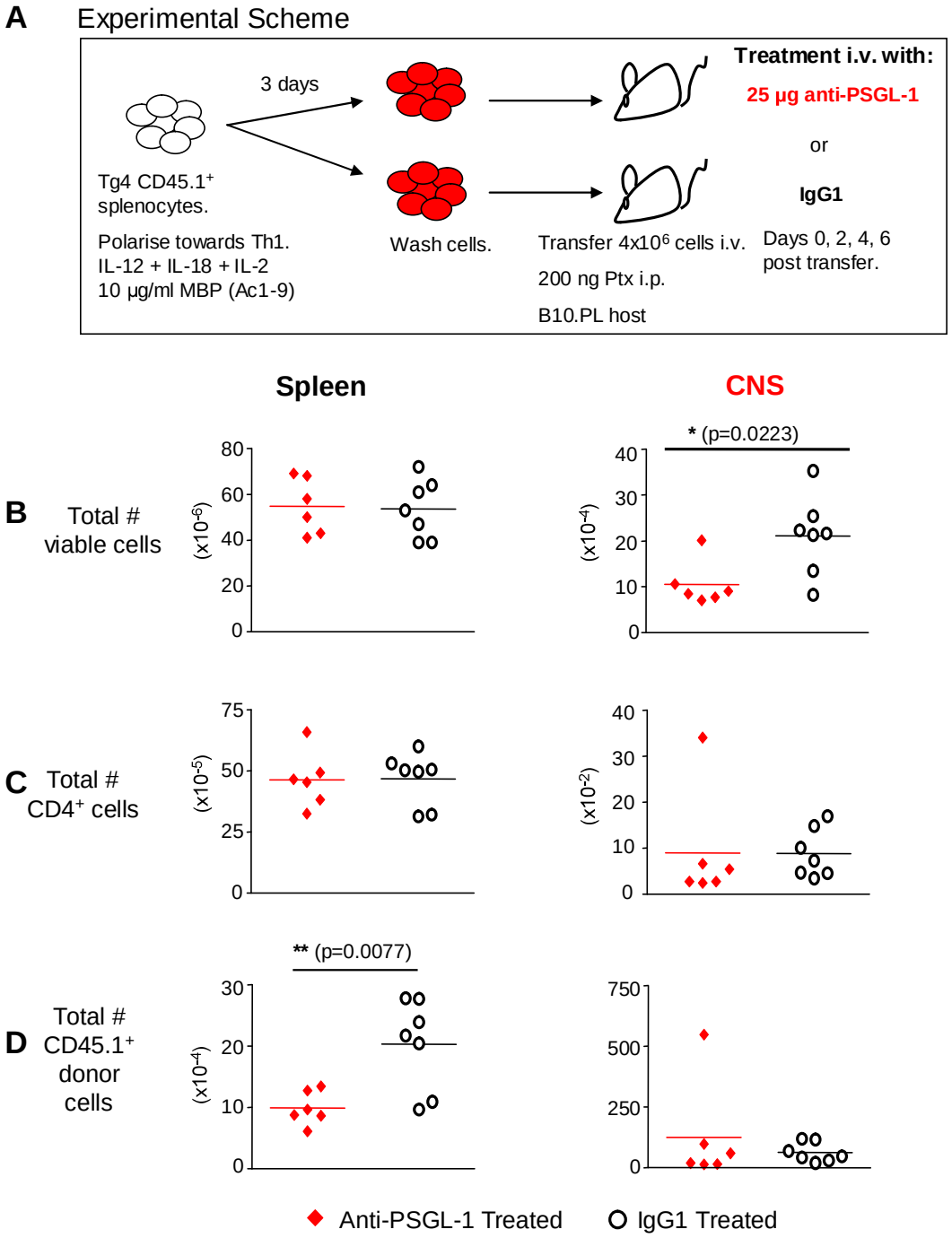

Figure 4.17 Effect of *in vivo* treatments with anti-PSGL-1 on entry of donor Tg4 Th1 cells into the periphery and CNS.

A, Experiment scheme; **B,** total number of viable cells; **C,** total number of $CD4^+$ T cells, and **D,** absolute number of $CD45.1^+$ Tg4 Th1 donor cells in the spleen (left) and CNS (right) at day 7 post-transfer after treatment with anti-PSGL-1 (♦) or IgG1 isotype control (○). Statistics performed with Mann Whitney Test (*p<0.05; **p<0.01; ***p<0.001). n=7 (IgG1 treated); n=6 (anti-PSGL-1 treated). Representative of one experiment.

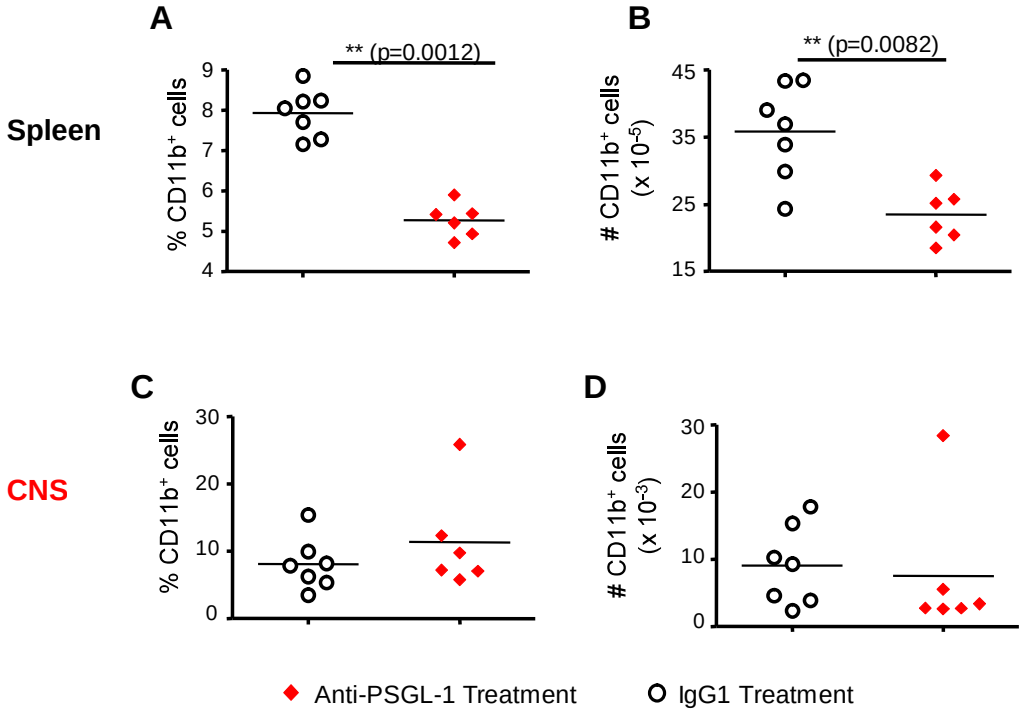

Figure 4.18 Effect of anti-PSGL-1 on the migration of CD11b$^+$ cells into the spleen and CNS during EAE.

A, Percentage and **B,** absolute number of CD11b$^+$ cells in the spleen; **C,** percentage and **D,** absolute numbers of CD11b$^+$ cells in the CNS. Samples taken at day 7 post-transfer after *in vivo* treatment on days 0, 2, 4 and 6 post-transfer with anti-PSGL-1 (♦) or IgG1 isotype control (○). Statistics performed with Mann Whitney Test (*p<0.05; **p<0.01; ***p<0.001). n=7 (IgG1 treated); n=6 (anti-PSGL-1 treated). Representative of one experiment.

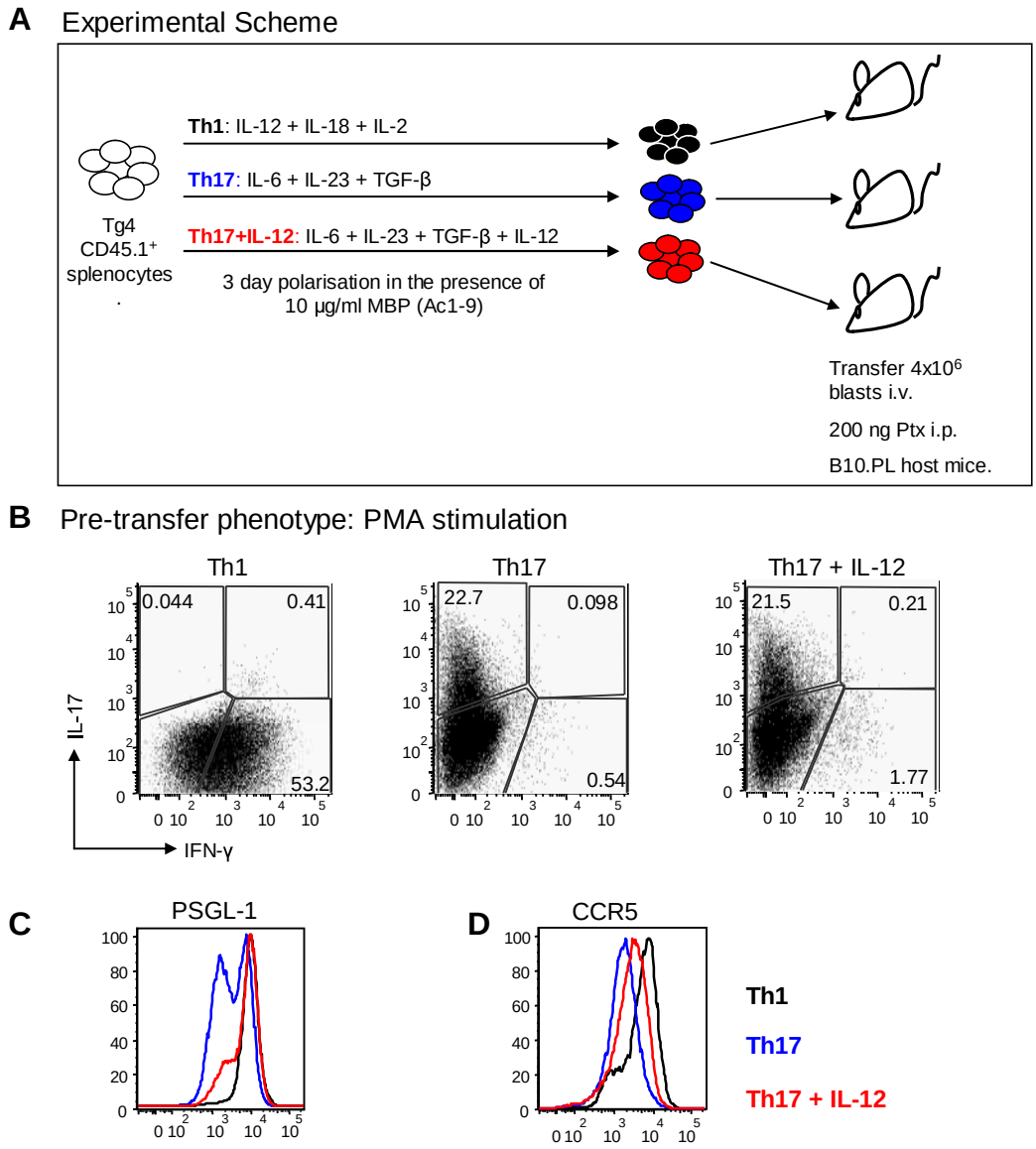

Figure 4.19 IL-12 increases the expression of PSGL-1 on Th17 cells *in vitro*.

A, Experiment scheme; **B**, Pre-transfer phenotype of Th1 and Th17 cells polarised in the presence/absence of IL-12, gated on $CD4^+$ cells showing IFN-γ and IL-17 production; Gated on $CD4^+$ cells **C,** PSGL-1 expression and **D,** CCR5 expression, on Th1 (**black**), Th17 (**blue**) and Th17+IL-12 (**red**) cells. Representative of one experiment.

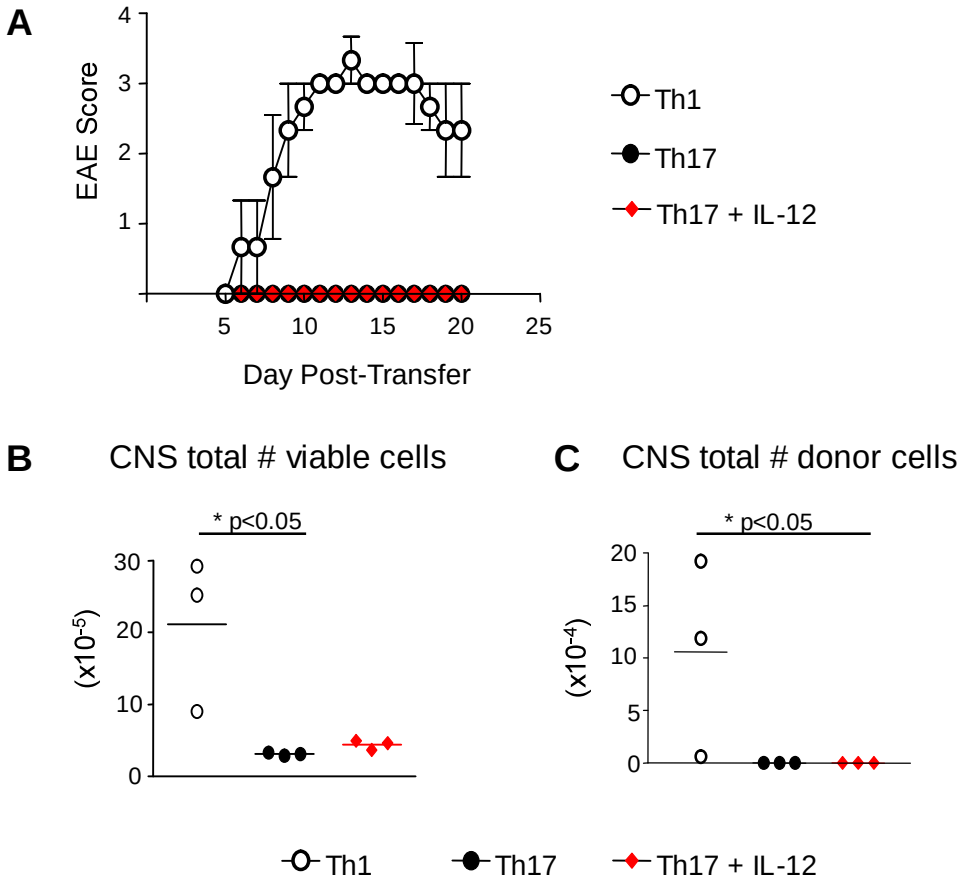

Figure 4.20 Increased PSGL-1 expression on Th17 cells does not confer pathogenicity.

A, Mean EAE scores versus days post transfer of mice with Th1 (○), Th17 (●) and Th17 + IL12 (♦) cells. **B**, Total number of lymphocytes in the CNS at day 20 post-transfer; **C**, Total number CD45.1^{+} donor cells in the CNS at day 20 post-transfer. Disease incidence: Th1 (3/3), Th17 (0/3) and Th17+IL-12 (0/3). Results representative of 2 experiments. Statistics performed using Kruskal Wallis test (KW p value: *p=0.0273 (B) and *p=0.0390 (C)) and Dunn's Multiple comparison post test (*p<0.05; **p<0.01; ***p<0.001). Error bars represent mean ± standard error. Representative of one experiment.

5 The requirements of IFN-γ, IL-17 and TNF-α in EAE

5.1 Introduction

As shown in Chapter 3, Th1 cells were able to infiltrate the CNS and induce EAE whereas Th17 cells could not (O'Connor et al., 2008). One clear difference between the pathogenic Th1 cells and the non-pathogenic Th17 cells is their production of their signature cytokines, IFN-γ and IL-17 respectively. IFN-γ has been shown to have a pivotal role in promoting a pro-inflammatory response by the activation of macrophages, upregulation of adhesion molecules and the upregulation of MHC molecules (Steeg et al., 1982; Basham and Merigan, 1983; Dore-Duffy et al., 1996).

However, despite its obvious role in promoting a pro-inflammatory environment, the involvement of IFN-γ in EAE induction is not straightforward and it appears to have a protective effect (Ferber et al., 1996; Willenborg et al., 1996). Mice with a disrupted IFN-γ gene are still susceptible to MBP-induced EAE (Ferber et al., 1996). Similarly, mice lacking the ligand binding chain of the IFN-γR develop more severe MOG-induced EAE (Willenborg et al., 1996). In addition to this, the transfer of MOG-specific IFN-γR-deficient cells, into IFN-γR-deficient hosts results in severe EAE with no recovery. In contrast, if the cells are transferred into IFN-γR-competent hosts, these mice develop severe EAE as well, but recover from disease (Willenborg et al., 1996). Treatments with anti-IFN-γ have been shown to lead to enhanced EAE development (Billiau et al., 1988; Lublin et al., 1993). Reciprocally, the administration of IFN-γ to EAE mice resulted in the suppression, or amelioration of disease (Billiau et al., 1988; Voorthuis et al., 1990). This evidence all suggested IFN-γ has a protective role in EAE.

Despite the possible beneficial roles of IFN-γ, EAE was still considered to be a Th1 mediated disease due to the inhibition of disease after anti-IL-12 treatments

(Leonard, Waldburger and Goldman, 1995; Bright et al., 1998a; Bright et al., 1998b) and the clear encephalitogenic potential of Th1 cells (Ando et al., 1989). In addition to this, IL-12-deficient mice exhibited resistance to EAE induction (Segal, Dwyer and Shevach, 1998). However, since the discovery of IL-23, a cytokine that shares its p40 chain with IL-12 (Oppmann et al., 2000), and the discovery that IL-23 had a dominant role to play in EAE pathology (Cua et al., 2003; Chen et al., 2006a), this view-point shifted towards Th17 cells being the key pathogenic lineage (Langrish et al., 2005; Park et al., 2005). Studies into the role of IL-17 in autoimmune inflammation are extensive, some showing evidence for its pro-inflammatory effects (Komiyama et al., 2006) and others showing evidence for IL-17 not being the key pro-inflammatory cytokine (Haak et al., 2009).

Overall, studies have therefore suggested that, although both Th1 and Th17 cells appear able to induce disease, neither of the signature cytokines is required for the induction of EAE (Willenborg et al., 1996; Haak et al., 2009). Therefore, perhaps another pro-inflammatory cytokine, for example TNF-α, which is produced by both Th1 and Th17 cells, is required for the induction of disease and this was investigated here.

'Atypical' or 'non-classical' EAE has been described recently in different models of EAE, including BALB/c IFN-γ-deficient mice, as well as spontaneous atypical EAE in TCR transgenic $RAG^{-/-}$ IFN-γ-deficient mice (Muller, Pender and Greer, 2000; Abromson-Leeman et al., 2004; Wensky et al., 2005). Notably, it is often associated with an absence of IFN-γ. Classical EAE is characterised by ascending clinical deficit, starting with tail paralysis and leading to forelimb paralysis which may or may not resolve. This appears to be caused by inflammation especially within the spinal cord, and little involvement of the brainstem and cerebellum (Muller, Pender and Greer, 2000; Abromson-Leeman et al., 2004). Atypical EAE is characterised by inflammation within the cerebellum and/or brainstem, with very limited spinal cord involvement and therefore presents as axial rotatory movement and disturbance in balance or co-ordination (Muller, Pender and Greer, 2000; Abromson-Leeman et al., 2004).

5.1.1 Aims

The aims of this final chapter were to investigate the requirements of a) IFN-γ, as the signature cytokine of pathogenic Th1 cells, b) TNF-α, as a pro-inflammatory cytokine produced by both pathogenic Th1 cells and non-pathogenic Th17 cells, and c) IL-17 as the signature cytokine of non-pathogenic Th17 cells, in EAE induction.

5.1.2 Approach

Both the C57BL/6/MOG and the Tg4/MBP(Ac1-9) models were used to investigate the requirements of IFN-γ in EAE. Firstly, pMOG-induced active EAE was induced in WT C57BL/6 and IFN-γKO mice to verify the more severe disease observed by others in IFN-γKO mice. The pathogenic ability of MOG-responsive WT and IFN-γKO Th1 cells was determined by passive transfer into WT hosts. This involved pMOG/CFA immunisation and the subsequent polarisation of Th1 cells *in vitro*. The C57BL/6 IFN-γKO mice were also crossed with Tg4 CD45.1 mice, to generate Tg4 IFN-γKO CD45.1 mice. This allowed the investigation of the requirements for IFN-γ using naïve T cells activated for the first time *in vitro* in the presence of MBP (Ac1-9). Polarised Th1 cells from Tg4 or Tg4 IFN-γKO mice were then transferred into B10.PLxC57BL/6 host mice. The role of TNF-α was investigated by antibody blockade using the Tg4 Th1 passive transfer model as described above.

IL-17FKO (H-2^b) mice were obtained and crossed with IFN-γKO H-2^b to generate IL-17FKO/IFN-γKO double knockout mice for planned use as donors for adoptive transfer. Delays in the generation of these double knockout mice prevented the description of their use in this thesis.

5.2 Results

5.2.1 IFN-γKO mice are susceptible to EAE and develop a more severe chronic form of disease compared to WT mice

Initial experiments to verify the reported protective role of IFN-γ (Ferber et al., 1996; Willenborg et al., 1996) used actively induced disease in IFN-γKO versus wild-type C57BL/6 mice (Figure 5.1 A). The IFN-γKO mice showed significantly increased susceptibility to the induction of EAE (Figure 5.1 B). This initial experiment was repeated and spleen, lymph node and CNS samples taken from both groups at days 6, 13 and 21 post-immunisation, see Figure 5.2 A for the EAE scores. At day 6, there was no significant difference in the total number of viable cells or number of $CD4^+$ T cells in the spleen or inguinal lymph nodes between the WT and IFN-γKO groups (Figure 5.2 B, C). There was an increased percentage of $CD4^+$ T cells in the spleen of the IFN-γKO mice, but this did not translate to increased absolute numbers of $CD4^+$ T cells due to these mice having slightly lower total cells in the spleen (Figure 5.2 B). Similarly, there was no evidence for a greater infiltrate in the CNS of the IFN-γKO mice either (Figure 5.2 D).

At day 13 post-immunisation (the peak of disease in WT mice) there were no significant differences between the WT and IFN-γKO in the spleen (Figure 5.3A). In the inguinal lymph nodes, there were significantly higher proportions and absolute numbers of $CD4^+$ T cells in the IFN-γKO mice (Figure 5.3 B). In the CNS there was a significantly higher percentage of $CD4^+$ T cells in the IFN-γKO mice compared to the WT. However, this did not translate to higher absolute numbers of $CD4^+$ T cells in the CNS of the IFN-γKO mice (Figure 5.3 C).

At day 21 post-immunisation, (corresponding to the recovery phase in the WT mice), significantly fewer total cell numbers were observed in the spleen of the IFN-γKO mice compared to the WT mice, and this also correlated with significantly fewer $CD4^+$ T cells in the spleen (Figure 5.4 A). The same trend was seen in the inguinal lymph nodes and CNS as was observed at day 13 post-immunisation (Figure 5.4 B,

C) with no differences in absolute number of $CD4^+$ T cells within the CNS between the WT and IFN-γKO mice, despite the percentage of $CD4^+$ cells being significantly higher in the IFN-γKO mice.

5.2.2 Comparable numbers of FoxP3$^+$ Treg in the CNS of WT and IFN-γKO EAE mice

The resolution of classical EAE correlates with an accumulation of Treg in the CNS leading to the suppression of inflammation (McGeachy, Stephens and Anderton, 2005; O'Connor, Malpass and Anderton, 2007). Therefore, the prolonged disease observed in the IFN-γKO mice could be due to the lack of $FoxP3^+$ Treg-mediated suppression in the CNS as has been suggested (Wang et al., 2006). However, there were no significant differences in proportions of, or absolute numbers of, $FoxP3^+$ Treg in the CNS (Figure 5.5 A-C) or spleen and inguinal lymph nodes (data not shown) at days 6, 13, or 21 post-immunisation between the WT and IFN-γKO mice.

5.2.3 Significantly increased numbers of IL-17$^+$ T cells in the CNS of IFN-γKO mice compared to WT mice, in response to pMOG stimulation

Another explanation for the extended disease course observed in the IFN-γKO mice could be increased levels of pro-inflammatory cytokines within the target organ, compared to the WT mice. The level of IL-17 was investigated and, at day 13 post-immunisation, elevated numbers of $IL\text{-}17^+$ T cells were found in the CNS of IFN-γKO mice in response to pMOG re-stimulation (~11 % of the $CD4^+$ population in the IFN-γKO mice versus <1 % observed in the WT mice) (Figure 5.6 A, lower panel).

Importantly, the $FoxP3^+$ Treg in the CNS of the IFN-γKO mice also had a significantly increased frequency of $IL\text{-}17^+$ cells after re-stimulation with pMOG (Figure 5.6 A), compared to WT $FoxP3^+$ Treg. Quantitatively, there was a significantly higher percentage of pMOG-specific $IL\text{-}17^+$ cells in both the $FoxP3^-$

and FoxP3$^+$ populations in the CNS of the IFN-γKO mice compared to their WT counterparts (Figure 5.6 B). Secondly, the percentage of IL-17$^+$ cells within the FoxP3$^+$ and FoxP3$^-$ CD4$^+$ compartments was comparable in the IFN-γKO mice (Figure 5.6 B). 10% of the IFN-γKO Treg population were IL-17$^+$. It has been shown that in the FoxP3$^+$ Treg in the CNS (of WT mice) ~25% are pMOG-responsive (Korn et al., 2007; O'Connor, Malpass and Anderton, 2007). This would suggest that ~40% pMOG-responsive Treg are producing IL-17 here. This observed increase in IL-17 was specific to the CNS and was not observed in the spleen or lymph nodes (data not shown). These results indicated that the prolonged disease observed in the IFN-γKO mice is associated with the increased numbers of IL-17$^+$ cells in the target organ.

5.2.4 pMOG-reactive IFN-γKO Th1 cells are capable of inducing EAE

The next step was to determine the requirement for IFN-γ production by encephalitogenic T cells in the passive transfer models used in this thesis. Firstly the phenotype of IFN-γ-deficient 'Th1' cells was investigated (Figure 5.7 A). pMOG-reactive IFN-γKO Th1 cells produced higher percentages of IL-17 compared to the WT Th1 cells (Figure 5.7 B) as well as higher levels of TNF-α (Figure 5.7 C). The IFN-γKO Th1 cells could express T-bet although a clear T-bet$^-$ population was apparent whereas T-bet expression in WT Th1 cells was more uniform, as determined by flow cytometry analysis (Figure 5.7 D).

The pMOG passive transfer model was used to determine if the IFN-γ-deficient Th1 cells could induce disease (Figure 5.8 A). At the time of transfer the WT Th1 cells had 1% IFN-γ$^+$ cells. Interestingly, in this experiment the IFN-γKO Th1 cells had ~14% IL-17$^+$ cells (as compared to 0.5% IL-17$^+$ WT Th1 cells) (Figure 5.8 B). WT Th17 cells had ~6% IL-17$^+$ and again, in the absence of IFN-γ, the IFN-γKO Th17 cells had a higher percentage of IL-17$^+$ cells (~19%) (Figure 5.8 B) as well as higher MFI values of the IL-17$^+$ population, indicating higher levels of IL-17 production. The level of IFN-γ and IL-17 production was also verified by ELISA using supernatants from the 3 day *in vitro* culture of Th1 polarised cells. The WT Th1 cells

produced greater than 100 ng/ml IFN-γ (the maximum limit of detection of the ELISA) (Figure 5.8 C, left). IL-17 was detectable in the supernatants from WT Th1 cells, but more was produced by the IFN-γKO Th1 cells (Figure 5.8 C, right).

Both WT and IFN-γKO Th1 cells were able to induce EAE. Despite the high IL-17 production in both Th17 populations, these were once again unable to induce disease (Figure 5.9 A). At day 12 post-transfer donor cells were evident in the spleens of each group, but only Th1 donor cells were enriched within the CNS (Figure 5.9 B). The WT Th1 donor cells had highly upregulated IFN-γ production in the CNS whereas the IFN-γKO Th1 cells had highly upregulated IL-17 production in the CNS (Figure 5.9 C). This was after PMA re-stimulation, and is therefore representative of non-specific cytokine production and not pMOG-specific production showing that the cells were capable of producing these cytokines on activation. The high number of IL-17^{+} cells in the CNS of the IFN-γKO Th1 transferred cells could have an influence on the disease severity. However, an increased disease severity by the IFN-γ-deficient cells was not evident from this experiment.

All mice showed signs of classical, rather than atypical, EAE.

5.2.5 TCR transgenic Tg4 IFN-γ KO Th1 cells also express T-bet to the same level as the Tg4 WT Th1 cells

The phenotype of Tg4 IFN-γKO Th1 cells was determined by flow cytometry and RT-qPCR analysis (Figure 5.10 A). As expected, the WT Th1 cells (denoted here as IFN-γ$^{+/+}$) had the highest frequency of IFN-γ^{+} cells, with the IFN-γ heterozygous cells (IFN-γ$^{+/-}$) having less and the IFN-γKO Th1 cells (IFN-γ$^{-/-}$) none (Figure 5.10 B, top). There were consistently slightly increased numbers of IL-17^{+} cells in the IFN-γ$^{-/-}$ cells compared to the wild-type Th1 cells (Figure 5.10 B, top) although interestingly not to the levels observed in the C57BL/6/pMOG system. The frequency of TNF-α^{+} cells was comparable across the three genotypes (Figure 5.10 B, bottom).

The IFN-$\gamma^{-/-}$ Th1 cells expressed T-bet (68.6% T-bet^{+}), albeit to a slightly decreased level compared to the IFN-$\gamma^{+/+}$ (82% T-bet^{+}) and IFN-$\gamma^{+/-}$ (76.6% T-bet^{+}) populations (Figure 5.10 C, D). This mimicked the T-bet expression observed on the pMOG-reactive IFN-γKO Th1 cells shown earlier (Figure 5.7 D). T-bet and RORγt mRNA expression was also determined on these cell populations, using RT-qPCR. T-bet was found to be upregulated to the same level in the three Th1 populations as compared to the Th17 control as well as being upregulated on a Th0 population (Figure 5.10 E). Th1 IFN-$\gamma^{+/+}$ cells did not have upregulated levels of RORγt expression (Figure 5.10 E). Interestingly, RORγt expression was upregulated two-fold on the Th1 IFN-$\gamma^{+/-}$ cells and upregulated four fold on the Th1 IFN-$\gamma^{-/-}$ cells compared to a Th0 control, but this was still much lower than the highly upregulated expression levels on IFN-$\gamma^{+/+}$ Th17 polarised Tg4 cells (Figure 5.10 E).

5.2.6 Tg4 IFN-γKO Th1 cells induce both classical and atypical EAE

The pathogenic potential of the Tg4 IFN-γKO Th1 cells was assessed (Figure 5.11 A). The IFN-γKO Th1 cells showed a slightly higher percentage of IL-17^{+} cells compared to the WT Th1 cells (Figure 5.11 B, top) and comparable frequencies of TNF-α^{+} cells (Figure 5.11 B, bottom). Both populations were T-bet^{+} (Figure 5.11 C). Both WT and IFN-γKO Th1 cells induced classical EAE (Figure 5.11 D). Interestingly, however, the IFN-γKO Th1 cells were also able to induce atypical EAE (Figure 5.11 E) whereas the WT Th1 cells did not. Of the ten mice that received IFN-γKO Th1 cells, 50% developed classical EAE and 50% developed atypical EAE, whereas all the mice with WT Th1 cells developed classical disease. Mice that developed classical disease did not show signs of atypical disease and vice versa.

5.2.7 Donor cell infiltration and inflammation occur specifically in the brain during atypical EAE but not the spinal cord

Atypical EAE is characterised by inflammation within the brain-stem and cerebellum and no involvement of the spinal cord (Wensky et al., 2005). To determine whether this was the same in our Tg4 passive transfer model, spleen, brain and spinal cord samples were taken at day 11 post-transfer. There was no significant difference in the total number of viable cells, the proportion and total number of $CD4^+$ T cells, or the proportion and total number of donor cells in the spleen (Figure 5.12 A) or brain of mice with classical versus atypical EAE (Figure 5.12 B). However, there were significantly fewer total cells in the spinal cord of mice with atypical EAE. This correlated with significantly fewer total $CD4^+$ T cells and fewer donor $CD45.1^+$ cells despite no difference in the proportion of donor $CD45.1^+$ cells between the two groups (Figure 5.12 C). In summary, during classical EAE donor cell infiltration and inflammation appeared to be observed in both the spinal cord and the brain (Figure 5.13 A). In contrast during atypical EAE, the donor T cells were located in the brain specifically and not the spinal cord (Figure 5.13 A).

5.2.8 Increased frequencies of IL-17^+ cells in the donor cells in the brains of mice with atypical EAE

The cytokine production by the donor T cells at day 11 post-transfer was investigated after an over-night re-stimulation with MBP (Ac1-9). Of the twelve mice sampled at this time-point, seven had exhibited classical disease and five atypical. The remaining mice (all classical EAE) were left to run the course of the disease. Of the seven mice exhibiting classical disease, five had received WT Th1 cells and two had received IFN-γKO Th1 cells.

In response to peptide stimulation there were approximately 8% IFN-γ^+ donor cells in the spleen, with higher frequencies apparent in the spinal cord and brain (~20%) in the mice with classical EAE (Figure 5.13 B). The mice that exhibited atypical EAE had negligible proportions of IFN-γ^+ cells due to the donor cells, all being IFN-γKO

(Figure 5.13 B). Comparable percentages of donor T cells were TNF-α^+ in the spleen and brain of mice with classical EAE induced by WT or IFN-γKO cells versus atypical EAE (Figure 5.13 B). Significantly lower percentages of donor cells were TNF-α^+ in the spinal cord of mice with atypical EAE compared to those with classical disease. However, this was most likely due to technical issues, given the very low cell numbers in these samples. The percentage of IL-17^+ cells in the spleen was low in both classical and atypical disease. In the spinal cord, percentages of IL-17^+ cells were comparable between classical (induced by WT or IFN-γKO cells) versus atypical EAE. In the brain, the IFN-γKO donor cells produced IL-17 regardless of inducing classical or atypical disease, and the WT Th1 cells did not (Figure 5.13 B), however this was not statistically significant. The increased percentages of IL-17 cells in the brain in atypical EAE mice suggests perhaps the increased IL-17 production in the brain had led to the development of atypical EAE, however the two mice that developed classical EAE also had higher levels of IL-17 production in the brain which would argue against this idea.

5.2.9 Requirements of IL-17 for the induction of atypical EAE

The observation of increased IL-17^+ cells specifically in the brain of mice with atypical EAE, and not in the spleen, led to the assessment of whether this IL-17 production by the donor T cells was required for the induction of atypical disease. The experiment was repeated with the addition of the Miltenyi IL-17 secretion and detection assay which was used to deplete the IL-17^+ cells from a portion of Tg4 IFN-γKO Th1 polarised cells. Tg4 Th1, Tg4 IFN-γKO Th1 and Tg4 IFN-γKO Th1 IL-17^- cells were transferred separately into host mice (Figure 5.14 A). Pre-transfer analysis of the phenotype of the polarised cells showed they had a Th1 phenotype in terms of cytokine production and T-bet expression (Figure 5.14 B-D). The depletion step removed the majority of IL-17^+ cells from the Tg4 IFN-γKO Th1 population, reducing the IL-17^+ fraction from ~2% to 0.46%, generating a Tg4 'IFN-γKO IL-17^- Th1' population for transfer (Figure 5.14 E, F).

Tg4 Th1 cells induced classical EAE, and not atypical EAE (Figure 5.15 A). As seen in previous experiments, Tg4 IFN-γKO Th1 cells were able to induce classical or atypical forms of EAE (50% exhibited classical disease and 50% atypical disease). Interestingly, the Tg4 IFN-γKO Th1 IL-17$^-$ population also induced either classical or atypical EAE (37.5% exhibited classical disease and 62.5% atypical disease) (Figure 5.15 A).

At day 13 post-transfer 20 mice were sampled. Of these, 13 had classical EAE (8 induced with transfer of WT Th1 cells; 2 with transfer of Tg4 IFN-γKO Th1 cells; 3 with transfer of Tg4 IFN-γKO IL-17$^-$ Th1 cells); and 7 had atypical EAE (3 induced with transfer of Tg4 IFN-γKO Th1 cells; 4 with transfer of Tg4 IFN-γKO Th1 IL-17$^-$ cells). The accumulation of the donor T cells during classical and atypical EAE was also similar to previous experiments. Comparable numbers of total viable cells and donor cells were found between the two groups in the spleen (Figure 5.15 B, C, left). A significantly higher number of total viable cells and donor T cells were found in the brain of the mice with atypical disease (Figure 5.15 B, C, middle). This had not reached significance in previous experiments. A significantly lower number of viable cells and donor T cells were observed in the spinal cord of mice with atypical EAE compared to those with classical (Figure 5.15 B, C, right).

The cytokine production by the Tg4 IFN-γKO Th1 donor T cells that had been depleted of the IL-17$^+$ cells was tested to determine whether these cells could now produce IL-17 in response to MBP (Ac1-9) stimulation. Interestingly, both Tg4 IFN-γKO Th1 cell populations, regardless of whether they had been depleted of IL-17$^+$ cells or not, had raised frequencies of IL-17$^+$ cells in the brain in response to stimulation (Figure 5.16 A). The frequency of IL-17$^+$ cells in the Tg4 IFN-γKO populations was comparable and did not account for why some induced classical and not atypical disease. There was a trend towards lower frequencies of IL-17$^+$ cells in the Tg4 IFN-γKO cells that had induced classical disease, however this was not significant. Frequencies of IL-17$^+$ cells were negligible in the spleens of these mice (Figure 5.16 A). The Tg4 Th1 donor cells did not have any IL-17$^+$ cells, in the brain or spleen.

Comparable percentages of TNF-α^+ donor cells appeared in the spleen of mice receiving the different donor cell populations after stimulation with MBP (Ac1-9) stimulation (Figure 5.16 B). However, in the brain, the Tg4 Th1 donor cells had lower frequencies of TNF-α^+ cells compared to the Tg4 IFN-γKO donor cells although this was not significant (Figure 5.16 B). The Tg4 IFN-γKO Th1 cells that had induced classical EAE and not atypical EAE did not show significantly different cytokine production that could account for their induction of classical EAE. There was no significant difference in the percentage of IFN-γ^+ cells between the spleen and brain in the Tg4 Th1 transfers (Figure 5.16 C). The very low number of Tg4 IFN-γKO donor cells observed in the spinal cord made the comparison of cytokine staining between them and the WT cells in this tissue very unclear, so this has not been shown. Importantly, this cytokine production has clearly shown that despite depleting IL-17^+ cells prior to transfer, the Tg4 IFN-γKO cells were later able to produce IL-17 *in vivo* and/or on re-stimulation. This could either be an outgrowth of the 0.46% of IL-17^+ cells that were still present in the pre-transfer population, or could be previously IL-17^- cells becoming IL-17^+. However, it has not been possible to determine which population they came from in these experiments.

5.2.10 The presence of IL-18 in the polarising Th1-cocktail results in higher IFN-γ and TNF-α production by these cells, and increased severity and incidence of disease

As shown here, IFN-γ is not required for EAE induction and it has also been shown that IL-17 is not required for the induction of disease (Haak et al., 2009). However, both Th1 cells (O'Connor et al., 2008) and Th17 cells (Jager et al., 2009) appear able to induce EAE. Perhaps TNF-α, which is produced by both Th1 and Th17 cells, is required for disease induction by these cell populations. IL-18 was found to induce higher proportions of IFN-γ^+ and TNF-α^+ cells in Th1 populations and it was investigated as to whether this had an effect on their ability to induce EAE (Figure 5.17 A). The 'IL-12-alone' polarised Th1 cells had 14% IFN-γ^+ cells and 40% TNF-α^+ cells (Figure 5.17 B, left). The addition of IL-18 increased the proportion of IFN-γ^+ cells two-fold to 31%, and increased the proportion of TNF-α^+ cells to 57%

(Figure 5.17 B, right). Both populations induced classical EAE. However, the 'IL-12 + IL-18' polarised cells induced an apparent higher severity of disease although this was not statistically significant (Figure 5.17 D) compared to the 'IL-12-alone' cells. The increased production of IFN-γ and TNF-α by the 'IL-12 + IL-18' may have contributed to the enhanced disease severity. As it has been shown earlier that IFN-γ is not required for disease induction by Th1 cells, the observed increased severity of disease may be due to the increased proportion of TNF-α^+ cells, although this is not clear as other changes imposed by exposure to IL-18 were not determined.

5.2.11 Neutralising TNF-α during Tg4 Th1-driven EAE results in decreased severity of disease

To determine whether TNF-α had a role in EAE severity, a Tg4 Th1 passive transfer was done, and the host mice treated with a neutralising antibody against TNF-α **(**Figure 5.18 A). The donor Tg4 Th1 cells had a Th1 phenotype (Figure 5.18 B-C). A single treatment of a neutralising antibody against TNF-α (on the day of Tg4 Th1 cell transfer), resulted in a trend towards reduced disease severity as compared to the PBS-treated controls (although this was not statistically significant) (Figure 5.18 D) suggesting TNF-α has a key role in inducing severe EAE.

5.3 Discussion

The precise requirements for T cell derived factors to drive EAE remain controversial. It has clearly been shown here that Th1 cells are capable of infiltrating into the CNS and inducing EAE (O'Connor et al., 2008). This observation runs counter to the knowledge that IFN-γ, the signature cytokine of the Th1 lineage, is not required for the induction of disease. This led to the decision to revisit the requirement for IFN-γ using our C57BL/6/pMOG and Tg4/MBP(Ac1-9) passive transfer models. The endeavour was to use the traceable models developed here to shed new light on the reasons for exacerbated disease in the absence of IFN-γ.

Previously, it has been shown that disruption of IFN-γ signalling, by genetically targeting either IFN-γ itself or the IFN-γR, resulted in more severe and prolonged

EAE (Ferber et al., 1996; Willenborg et al., 1996; Willenborg et al., 1999). Here, pMOG-induced EAE was compared between WT C57BL/6 and IFN-γKO mice on the same background. A more severe and chronic form of EAE was observed in the IFN-γKO mice compared to the WT mice. However, this did not correlate to higher absolute numbers of inflammatory $CD4^+$ T cells in the CNS at a pre-clinical time-point, the peak of disease, or at the point where the WT mice were recovering. There was a significant increase in the number of IL-17^+ cells in the CNS of IFN-γKO mice which could contribute to the more severe disease observed, and is suggestive of upregulated IL-17 response in the absence of IFN-γ which has not been directly reported. Another simple explanation for the more severe disease observed in the IFN-γKO mice could be decreased levels of $Foxp3^+$ Treg in the CNS as has been observed previously (Wang et al., 2006). IFN-γ has been suggested to be required for the conversion of $CD4^+$ $CD25^-$ cells to $CD4^+$ $CD25^+$ Treg, and reduced frequency and function of Treg was observed in IFN-γKO mice (Wang et al., 2006). This was thought to contribute to the enhanced disease severity observed in these mice compared to the WT mice. However, no evidence for such an explanation was obtained in this study as $FoxP3^+$ Treg frequencies were comparable between WT and IFN-γKO EAE mice.

Surprisingly, despite not finding differences in the frequency or number of $FoxP3^+$ Treg in the CNS, a significant proportion of the $Foxp3^+$ cells in the CNS of the IFN-γKO mice were IL-17^+, whereas this was not observed either in the spleen of the same mice (data not shown), or in the CNS or spleen of the WT mice. This effect was also not observed in the case of IFN-γ or TNF-α (data not shown). This suggests that the $FoxP3^+$ Treg in this setting are able to switch from a regulatory phenotype to a pro-inflammatory phenotype, which could have a large impact on the current interest in using Treg for cell therapy in autoimmune disease. The observation of the apparent Treg plasticity in the IFN-γKO mice is also supported by the observation that IFN-γ prevents Treg conversion to IL-17 production *in vitro* (R. O'Connor, personal communication). This is supported by a recent observation in human T regulatory cells (Beriou et al., 2009). In this case a population of human $FoxP3^+$ Treg began producing IL-17 if stimulated in the presence of pro-inflammatory cytokines.

The Foxp3$^+$ IL-17$^+$ cells obtained could exhibit both suppressive and pro-inflammatory effects depending on the cytokine environment (Beriou et al., 2009).

Treg are becoming an attractive route of therapy for autoimmune disease. However, care should therefore be taken in determining whether Treg have a fixed phenotype or whether they can switch from a regulatory to an inflammatory role in particular settings as has been shown (Yang et al., 2008c; Komatsu et al., 2009), as this would clearly have a negative impact on their use for therapeutics. In the same way, it has been recently shown that other CD4$^+$ T cell subsets e.g. Th17 and Th2 cells, exhibit plastic phenotypes (Bending et al., 2009; Hegazy et al., 2010).

The phenotype of IFN-γKO Th1 cells and their pathogenic capabilities were further investigated. Both pMOG-reactive IFN-γKO Th1 cells and Tg4 IFN-γKO Th1 cells had increased capacity for IL-17 production compared to WT cells, an attribute which has not been directly reported before. The Tg4 IFN-γKO Th1 cells also exhibited raised RORγt expression. Several molecules are known to directly restrict the differentiation of Th17 cells including IL-2 through the activation of STAT5 (Laurence et al., 2007) and nuclear receptor peroxisome proliferators-activated receptor-γ (PPARγ) (Klotz et al., 2009). The neutralisation of IL-4 and IFN-γ during Th17 differentiation is thought to increase the proportion of IL-17$^+$ cells produced by inhibiting the Th2 and Th1 pathways respectively, suggesting that IFN-γ inhibits IL-17 production (Harrington et al., 2005; Park et al., 2005) and this is routinely used in *in vitro* Th17 differentiation by some groups. Here the absence of IFN-γ appears to be sufficient to allow IL-17 and RORγt expression, even under Th1 polarising conditions, suggesting that IFN-γ has a suppressive effect on IL-17 production in cells that would otherwise be producing IFN-γ.

pMOG-reactive IFN-γKO Th1 cells expressed T-bet, the key transcription factor of the Th1 lineage (Szabo et al., 2000), albeit to a slightly decreased level compared to the WT cells. This is perhaps not surprising as T-bet expression can be upregulated through IL-12, which is present in the polarising cytokine mix. The same was observed in the Tg4 IFN-γKO Th1 cells, which expressed T-bet (albeit at slightly

lower levels) as did the WT Th1 cells both at the protein and mRNA level. T-bet is known to be the master regulator of Th1 differentiation, as it was first reported to be specifically expressed on Th1 cells. Ectopic expression of T-bet in Th2 cells resulted in suppression of the Th2 cytokine responses, and up-regulation of IFN-γ (Szabo et al., 2000; Lovett-Racke et al., 2004). It has been suggested that T-bet expression is dependent on IFN-γ and STAT1 signalling (Afkarian et al., 2002), however, data presented here shows that T-bet can still be induced in IFN-γKO cells polarised towards a Th1 phenotype. Although IFN-γ clearly does play an important role in the regulation of T-bet expression, T-bet is clearly not absent from IFN-γKO or STAT-1KO mice (data presented here; (Lighvani et al., 2001)) indicating that there are other IFN-γ-independent regulators of T-bet expression, perhaps directly through IL-12 signalling (Schultz et al., 2009).

It has previously been shown that TCR transgenic $RAG^{-/-}$ mice lacking IFN-γ are susceptible to spontaneous atypical EAE (Wensky et al., 2005). Classical and atypical EAE present very distinct signs of disease; classical EAE being characterised by spinal cord inflammation leading to increasing paralysis, from the tail forwards, and atypical EAE being characterised by inflammation specifically within the brain-stem and cerebellum leading to ataxic movements and loss of balance of the mice (Muller, Pender and Greer, 2000; Muller, Pender and Greer, 2005). To support the study of this, the involvement of brain-stem inflammation has also been observed in MS patients (Shibasaki, McDonald and Kuroiwa, 1981). Although classical EAE recapitulates some of the signs of MS, there are certain aspects that are not represented by the study of classical EAE. For example, MS patients display symptoms such as vestibular (balance) problems and vertigo (Sasaki et al., 1994; Williams, Roland and Yellin, 1997; Frohman et al., 2000), and the study of atypical EAE could allow for these other aspects of MS to be investigated.

Here, only classical EAE was induced by the transfer of WT Tg4 Th1 cells. However, the transfer of Tg4 IFN-γKO Th1 cells led to the induction of either classical or atypical forms of EAE. Tracking the donor T cells revealed their accumulation in both the spinal cord and the brain during classical EAE, but only in

the brain during atypical EAE, supporting previous reports that have shown inflammation during atypical EAE is specifically within the brain-stem and cerebellum (Wensky et al., 2005). It has been shown that co-transfer of WT Th1 cells together with the IFN-γKO Th1 cells is sufficient to restore inflammation within the spinal cord and the development of classical EAE (Lees et al., 2008) suggesting a protective effect of IFN-γ specifically within the brain.

The IL-17 production by the Tg4 IFN-γKO Th1 cells prior to transfer and the significantly higher levels of IL-17$^+$ cells in the donor cell population during atypical, but not classical, EAE suggested that in the absence of IFN-γ, IL-17 could drive pathology in the brain. Tg4 IFN-γKO Th1 cells depleted of IL-17$^+$ cells prior to transfer could still induce either classical or atypical EAE, to the same extent as the unsorted Tg4 IFN-γKO Th1 cells. However, when sorted and transferred Tg4 cells were recovered from the brain, these now contained measurable IL-17$^+$ cells. Therefore these results are not conclusive as to whether IL-17 is required for the induction of atypical EAE. The experiment needs to be refined in a cleaner system ideally through the transfer of IFN-γ-deficient-IL-17F-deficient cells together with the use of a neutralising antibody against IL-17A, to inhibit any IL-17 signalling.

The transfer of cells in the absence of both IFN-γ and IL-17 signalling has been achieved recently through the transfer of pMOG-reactive IFN-γKO effector cells into IL-17Rα-deficient mice (Kroenke, Chensue and Segal, 2010). This resulted in a shift from atypical EAE if the cells were transferred into WT hosts, to classical EAE, when transferred into IL-17Rα-deficient mice, suggesting that IL-17 is required (at least in a polyclonal setting) for the initiation of brainstem infiltrates and the induction of atypical EAE as opposed to classical EAE. This study also found that classical EAE, which could occur in the absence of both IFN-γ and IL-17, was dependent on GM-CSF as treatment with anti-GM-CSF led to the complete abrogation of disease (Kroenke, Chensue and Segal, 2010).

To support the idea that IL-17 may be required for the initiation of atypical disease, it has been shown, again in a polyclonal MOG system, that the induction of EAE in

transgenic mice in which IL-6 production is restricted specifically to the cerebellum, resulted in the induction of the atypical form of EAE, in contrast to the wild-type mice that developed classical EAE (Quintana et al., 2009). Inflammation was specifically located in the cerebellum of these mice, with no evidence of spinal cord involvement, suggesting site-specific IL-6 production re-directed the inflammatory response to the cerebellum only, away from the spinal cord where inflammation would normally occur (Quintana et al., 2009). This would suggest that IL-6 production specifically in the cerebellum would result in local inflammation, or in an increase in IL-17 signalling in that area, leading to the induction of atypical EAE. This adds weight to the suggestion that IL-17 signalling, or IL-17 itself, is required for the induction of atypical EAE within the polyclonal system. Interestingly, results here showed that transfer of polyclonal pMOG-responsive IFN-γKO Th1 cells, which had high percentages of IL-17$^+$ cells resulted in the development of classical EAE, not atypical EAE, whereas in the TCR transgenic model atypical EAE was induced. Furthermore in the Tg4 transfer model only ~50% developed atypical EAE, the rest showing classical EAE. It would be interesting to definitively determine if IL-17 signalling is also required for the induction of atypical EAE in the TCR transgenic system with the Tg4 IFN-γKO Th1 cells and this would be an important next step to investigate.

The literature surrounding the roles of IL-17 in the induction of inflammation is extensive, and somewhat contradictory. It has largely been accepted that IL-17 is required for the induction of EAE. Early evidence after the discovery of the Th17 lineage showed that IL-17A-deficient mice displayed a delayed onset and reduced severity of EAE, and the passive transfer of IL-17-deficient CD4$^+$ T cells led to the inefficient induction of EAE (Komiyama et al., 2006). It has also been shown that IL-17A has a more important role to play in the initiation of EAE, as opposed to IL-17F (Yang et al., 2008b). However, other recent evidence has shown that the over-expression of IL-17A did not exacerbate EAE severity, and secondly, the deficiency of either IL-17A, or its closely related family member, IL-17F, had a no significant impact on the severity of disease (Haak et al., 2009). Both IL-17A and IL-17F deficient mice are still fully susceptible to EAE induction, and blocking the

signalling of both to remove any compensatory effects also had no significant effects (Haak et al., 2009). This suggests that neither cytokine is required for the induction of disease, despite Th17 cells being able to induce EAE inflammation in numerous different settings (Langrish et al., 2005; Park et al., 2005). However, these early works that highlighted the pro-inflammatory role of 'Th17' cells used IL-23-induced 'Th17' cells which have since been shown to be unstable and not fully differentiated Th17 cells as IL-23 is only required for the stability of already differentiated Th17 cells (Stritesky, Yeh and Kaplan, 2008).

Other cytokines associated with Th17 cells have been shown not to have an important role in autoimmune inflammation. For example, IL-22 is produced by Th17 cells in an IL-23 dependent manner, and is co-expressed by the IL-17^+ cells. However, IL-22-deficient mice were observed to be fully susceptible to EAE (Kreymborg et al., 2007). IL-21 is another cytokine produced by Th17 cells however it has been found to be more important for the promotion of the Th17 lineage rather than for inducing pro-inflammatory responses. IL-21 is now known to be an autocrine regulator that promotes the production of IL-17 by Th17 cells and induces the expression of RORγt in Th17 cells (Nurieva et al., 2007; Wei et al., 2007). Interestingly though, the requirements of IL-21 in induction of EAE are controversial, with reports that IL-21-deficient mice are protected from disease induction, due to the impaired Th17 response (Nurieva et al., 2007). Contrasting with other studies reporting IL-21-deficient mice or IL-21R-deficient mice to have enhanced disease possibly due to a defect in the Treg mediated suppression of disease (Coquet et al., 2008; Liu et al., 2008a; Piao et al., 2008).

TNF-α has been shown to have a role in the development of EAE and is efficiently produced by both Th1 and Th17 cells. In MS, TNF-α has been reported to be upregulated in the acute lesions and chronic active lesions of MS patients, indicating that it has a role to play in the pathogenesis of MS (Selmaj et al., 1991). Treating host mice with a neutralizing antibody that blocks both LTα and TNF-α resulted in a reduced severity of disease by passive transfer (Ruddle et al., 1990). Similarly, using a SJL/J passive transfer model, it was demonstrated that treating mice with an

anti-TNF-α antibody resulted in the abrogation of disease (Selmaj, Raine and Cross, 1991). This correlates with results presented here which showed that, in the Tg4 passive transfer model, treatment of host mice with TN3-19.12, a monoclonal antibody against TNF (neutralises both TNF-α and TNF-β (LTα)) (Sheehan, Ruddle and Schreiber, 1989), gave rise to a lower severity of disease.

The role of lymphotoxin-α, which is produced by both Th1 and Th17 cells, has not been investigated in this project. LTα has been found to be upregulated in the lesions of MS patients in the same way as TNF-α suggesting that it may have a role in disease pathogenesis (Selmaj et al., 1991). Neutralizing both LTα and TNF-α as mentioned above led to a reduced severity of EAE by passive transfer (Ruddle et al., 1990). Neutralizing LTα led to the inhibition of EAE as well as collagen-induced arthritis (CIA) (Chiang et al., 2009). This inhibition of disease was due to depletion of IFN-γ-, IL-17- and TNF-α-producing cells.

However, in contrast to these apparent roles for TNF-α and LTα in autoimmune disease, it has also been reported that neither is required for EAE pathogenesis, through the generation of 129xC57BL/6 and SJL/J mice that are deficient for both TNF-α and LTα. Both double-knockout strains developed EAE after immunisation with mouse spinal cord homogenate, the SJL/J strain to a more severe extent (Frei et al., 1997). However, it could be that the entire knock-out of both genes could result in many more downstream processes being affected, resulting in differing results compared to the *in vivo* blocking through the use of a neutralizing antibody. To support this thought, another study involved the generation of another TNF-α-deficient mouse strain and these were found to be highly susceptible to MOG-induced EAE, once again suggesting that TNF is not required for EAE induction (Liu et al., 1998). Similarly, treatment with TNF, to either TNF-deficient mice, or wild-type mice, led to the development of less severe EAE, suggesting that it may indeed be beneficial and may be acting as an anti-inflammatory cytokine in this setting (Liu et al., 1998).

Anti-TNF therapy is widely used for the treatment of autoimmune diseases. After discovery that treatment with a monoclonal antibody against TNF had a beneficial

effect on CIA, this therapy was successfully translated to RA. Since then Etanercept, a TNF-receptor fusion protein and infliximab, an anti-TNF-α antibody, are known to be effective treatments for both RA and Crohn's disease (Moreland et al., 1997; Targan et al., 1997; Feldmann and Maini, 2010). Similarly, as mentioned above and in data presented here, anti-TNF treatments appear to decrease the severity of disease in EAE. Unfortunately, trials using anti-TNF therapies in MS patients resulted in worsening of disease (van Oosten et al., 1996; 1999) and it has even been shown, in rare cases, to cause development of MS-symptoms in patients treated for RA (Sicotte and Voskuhl, 2001). It is interesting that anti-TNF may be able to both inhibit inflammation in the joint in RA but increase de-myelination in the CNS. However, divergent roles of the TNF receptors in EAE have been identified which could shed light on the unexpected effects of anti-TNF therapy in MS patients. In the absence of TNFRI signalling, mice were resistant to MOG-induced EAE, however TNFRII-deficient mice exhibited exacerbated EAE suggesting TNFRII is required for the regulation of the immune response (or the integrity of CNS function) whereas TNFRI is involved in the initiation of inflammation (Suvannavejh et al., 2000). Notably both infiximab and etanercept block TNFRII signalling, possibly accounting for their exacerbating effects in MS (Franklin, 1999; Agnholt and Kaltoft, 2001; Ebert, 2009).

It is likely that all three cytokines investigated here produced by the encephalitogenic Th1 and Th17 cells have an interactive role to play with each other, and simply taking one out of the picture at a time, through the use of knock-out mice or neutralising antibodies does not allow for the full investigation of their pathogenic requirements. It would be interesting to determine whether $CD4^+$ T cells unable to elicit IFN-γ, IL-17 and TNF-α signalling are still able to induce disease. In addition, it could be that too much importance is being laid on the cytokine production by the $CD4^+$ encephalitogenic T cells. Perhaps it is other factors that confer their pathogenicity. It has been suggested that T-bet is a key regulator of pathogenicity in EAE (Bettelli et al., 2004; Lovett-Racke et al., 2004; Nath et al., 2006; Gocke et al., 2007; Yang et al., 2009). T-bet expression inducing pathogenicity would support the data shown here which indicates that T-bet^+ Th1-polarised cells, in the presence or

absence of IFN-γ are able to induce disease whereas T-bet$^-$ Th17 cells cannot. It has been shown that 'Th17' cells require T-bet expression for encephalitogenicity (Yang et al., 2009). In addition, TGF-β has been shown to suppress whereas IL-23 promotes Th17 differentiation and pathogenicity (Ghoreschi et al., 2010) and IL-23 has been shown to promote T-bet expression here (Chapter 3). The study of the down-stream products of Th1 and Th17 have not led to conclusive targets for therapy. Perhaps, the focus should shift more towards the study of the upstream pathways that lead to the production of encephalitogenic Th1 and Th17 cells, and determine what is key to their pathogenicity in this manner.

5.4 Conclusions

- The prolonged active EAE observed in the IFN-γKO mice was not due to a decrease in the proportion or number of $FoxP3^+$ Treg accumulating in the CNS of these mice.
- In the absence of IFN-γ, increased proportions of $IL\text{-}17^+$ cells were observed in the CNS, in the $FoxP3^-$ and $FoxP3^+$ populations.
- IFN-γKO cells polarised towards a Th1 phenotype *in vitro* express T-bet, and also produce IL-17 and these cells can transfer EAE. In contrast, Th17 cells which produce IL-17, but do not express T-bet, cannot transfer EAE.
- IFN-γKO cells exposed to their autoantigen during *in vivo* priming, induce only classical EAE (in both pMOG active EAE and pMOG passive EAE).
- In contrast, IFN-γKO cells exposed to their autoantigen for the first time *in vitro*, (in the Tg4 model), induce both classical and atypical EAE (with a 50:50 ratio).
- Classical EAE is characterised by inflammation in the spinal cord. In contrast, atypical EAE is characterised by donor cell migration and inflammation localised to the brain and not the spinal cord.
- Atypical EAE occurs in the absence of IFN-γ and may be dependent on IL-17 signalling.
- TNF-α has a role in disease pathogenesis as observed by the decrease in EAE severity in the absence of TNF-α signalling.

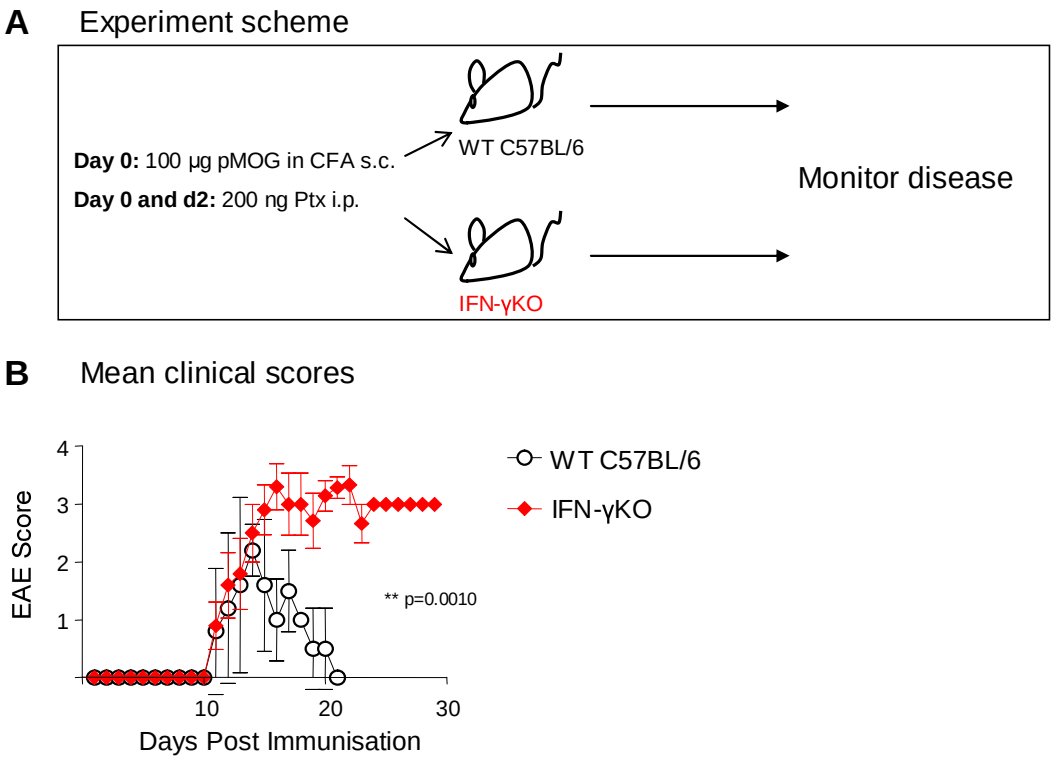

Figure 5.1 IFN-γKO mice develop more severe MOG-induced active EAE compared to wild-type C57BL/6 mice.

A, Experiment scheme; **B,** Mean scores of active EAE induced in wild-type mice (○) versus IFN-γKO mice (♦). Disease incidence: WT (7/10), IFN-γKO (10/11). The proportion of mice with severe EAE is significantly higher in the IFN-γKO group compared to the WT group (** p=0.0010) as determined using the Fisher's exact test. Data representative of one experiment. Error bars represent mean ± standard error.

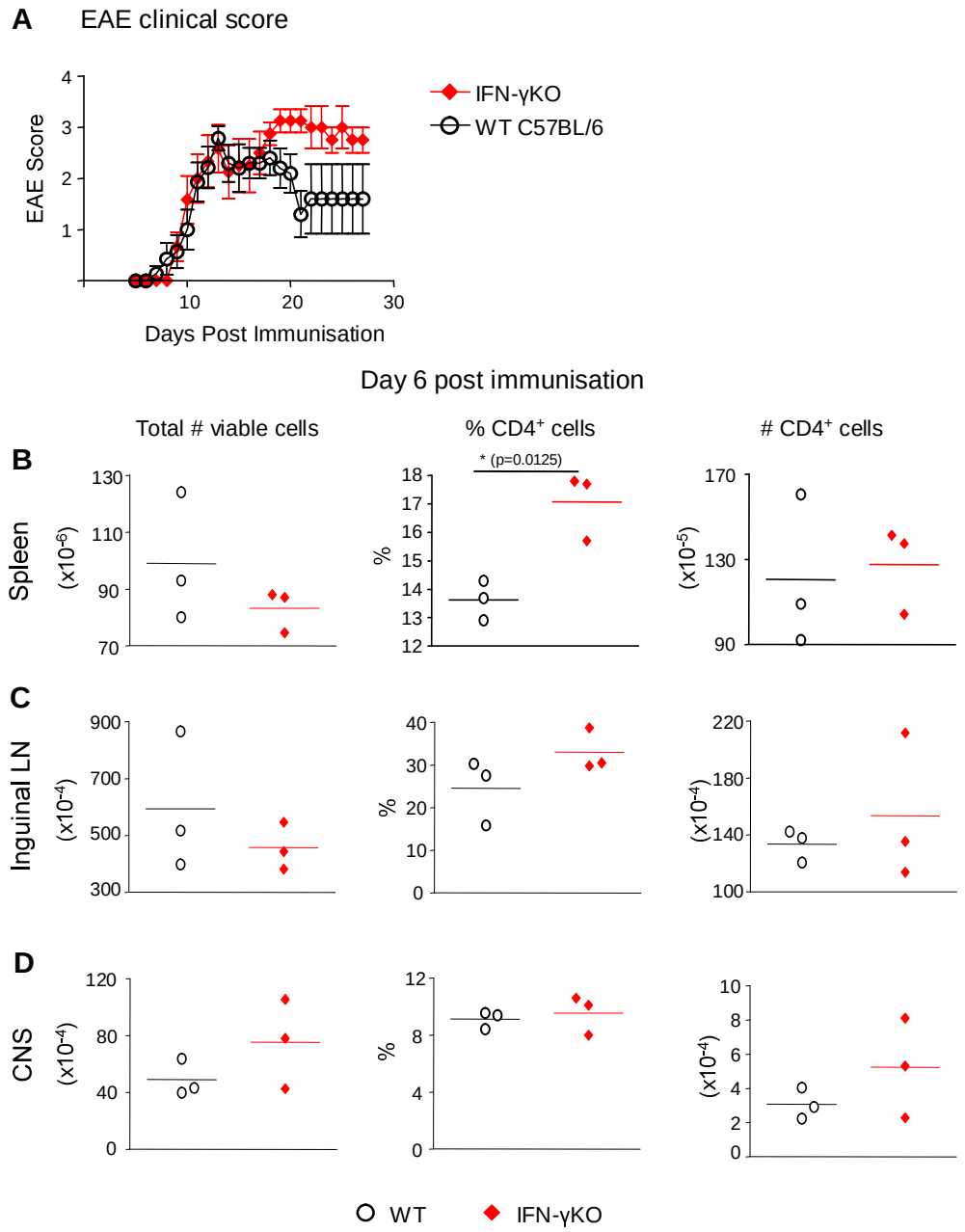

Figure 5.2 CD4$^+$ T cell infiltration into the periphery and CNS during active EAE in WT versus IFN-γKO mice, at day 6 post-immunisation.

A, Mean EAE scores after EAE induced in WT versus IFN-γKO mice. Day 6 post immunisation. Total number viable cells, percentage and absolute numbers of CD4$^+$ T cells in the **B,** Spleen; **C,** inguinal lymph nodes; and **D,** CNS between WT (○) and IFN-γKO (♦) mice. Disease incidence: WT (9/12), IFN-γKO (12/15). Statistical analysis performed with Mann Whitney Test ('*' represents: * $p<0.05$; ** $p<0.01$; *** $p<0.001$). Error bars represent mean ± standard error. EAE severity not significantly different between WT and IFN-γKO mice ($p=0.1131$ as determined by Fisher's exact test). n=3 for both groups. See Appendix 10 A for representative flow cytometry gating strategy.

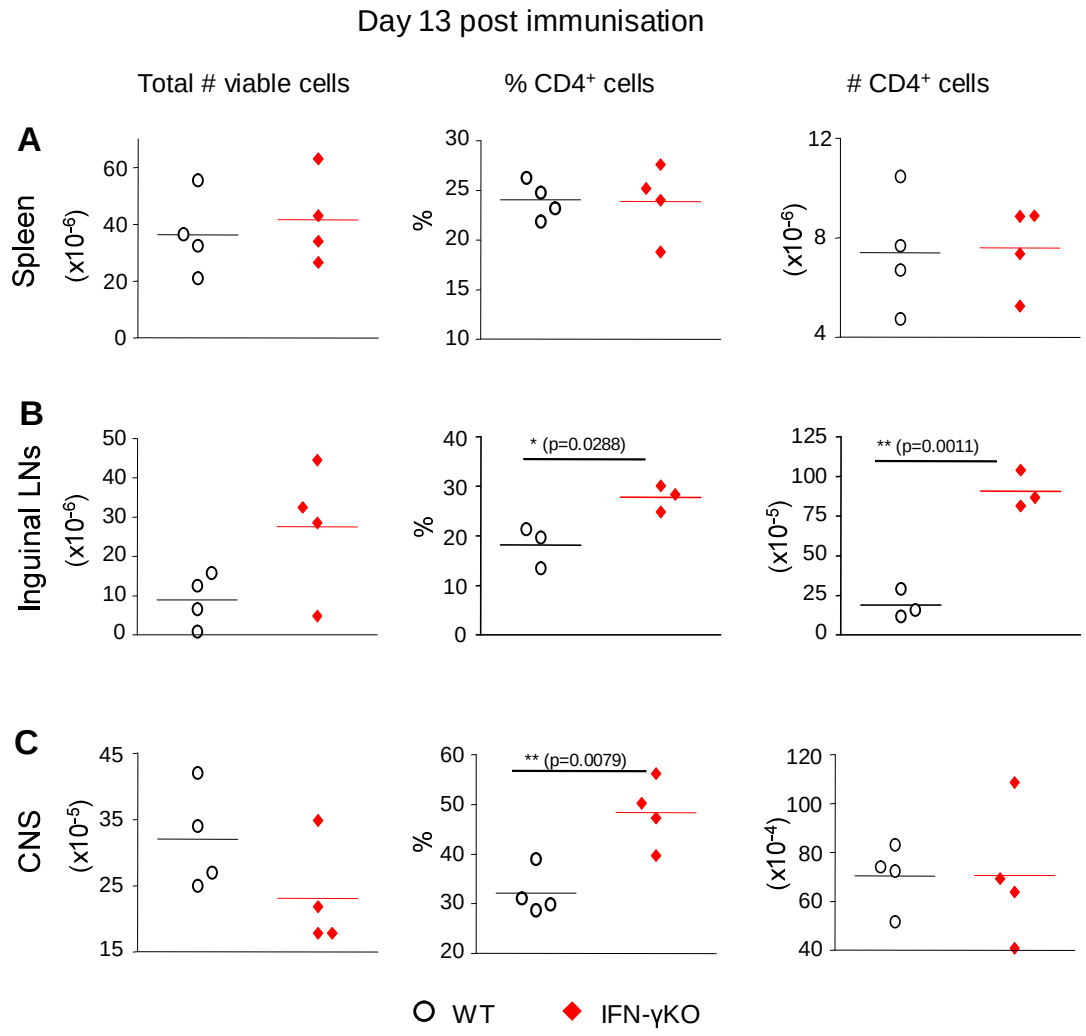

Figure 5.3 Day 13 post-immunisation (peak of disease): no significant difference in number of CD4$^+$ T cells in the CNS between WT and IFN-γKO EAE mice.

Total number viable cells, percentage and absolute number of CD4$^+$ T cells in the **A**, spleen; **B,** inguinal (draining) lymph nodes; and **C,** CNS between WT (○) and IFN-γKO (♦) mice. Statistical analyses performed with Mann Whitney Test ('*' represents: * $p<0.05$; ** $p<0.01$; *** $p<0.001$). n=4 for both groups.

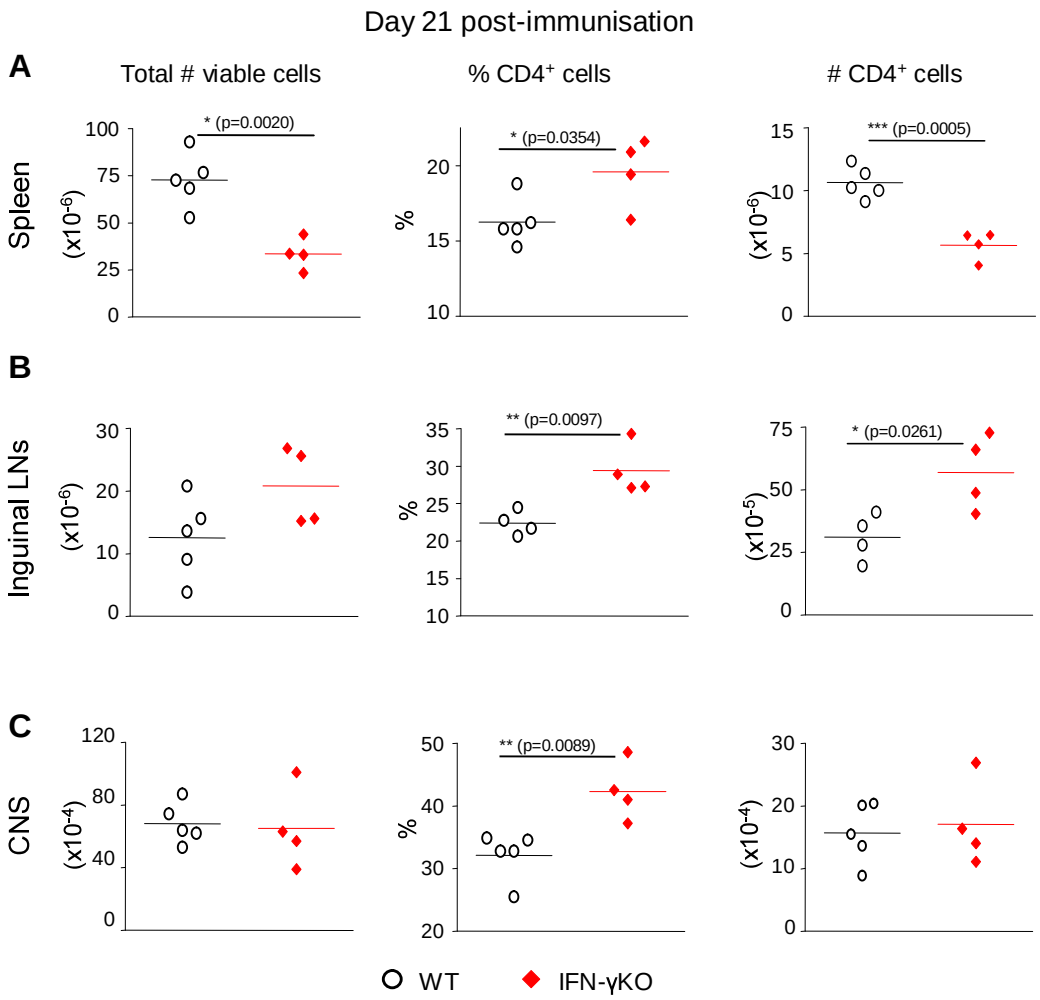

Figure 5.4 Day 21 post-immunisation: no significant difference in number of CD4$^+$ T cells in the CNS between WT and IFN-γKO mice.

Total number viable cells, percentage and absolute number of CD4$^+$ T cells in the **A**, spleen; **B,** inguinal (draining) lymph nodes; and **C,** CNS between WT (○) and IFN-γKO (♦) mice. Statistical analyses performed with Mann Whitney Test ('*' represents: * $p<0.05$; ** $p<0.01$; *** $p<0.001$). n=5 (WT); n=4 (IFN-γKO).

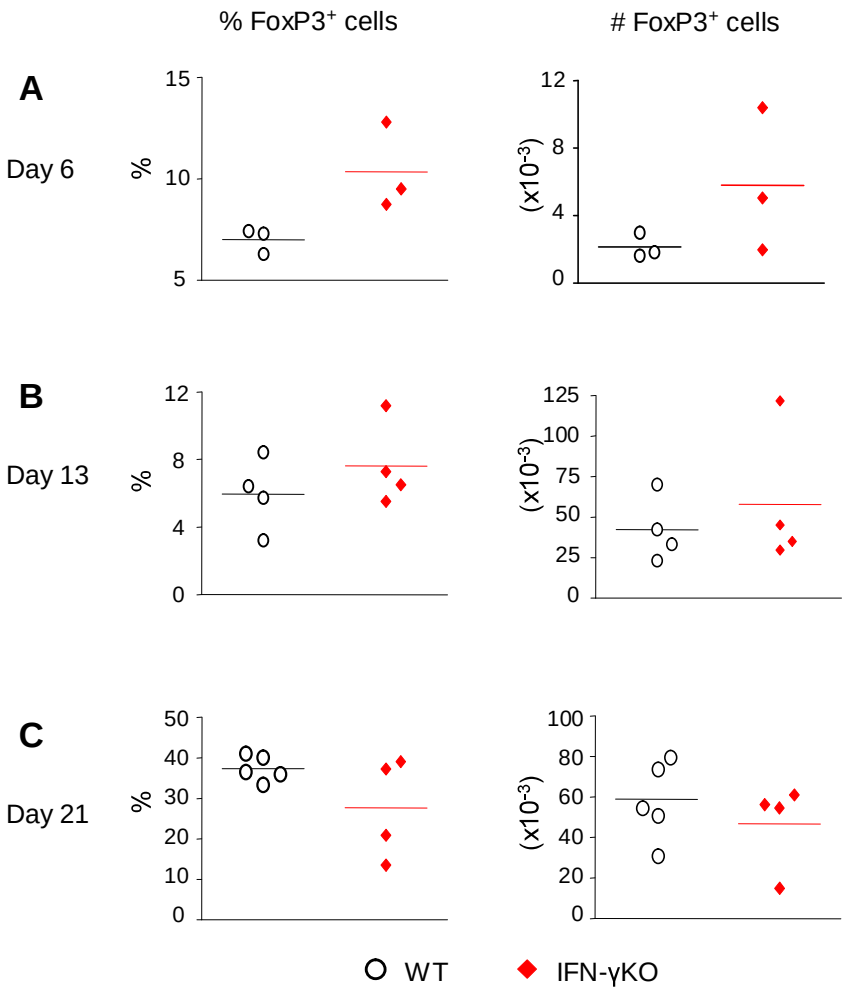

Figure 5.5 No significant difference in the percentage or absolute number of FoxP3$^+$ Treg in the CNS between WT and IFN-γKO EAE mice.

Ex-vivo analysis of FoxP3 expression on CD4$^+$ cells in CNS, showing percentage and absolute number of FoxP3$^+$ Treg in the CNS at **A,** day 6; **B,** day 13 and **C,** day 21 post-immunisation, between WT (○) and IFN-γKO (♦) mice. Statistical analyses performed with Mann Whitney Test ('*' represents: * $p<0.05$; ** $p<0.01$; *** $p<0.001$). See Appendix 10 B for representative flow cytometry gating strategy. For day 6, n=3 for both groups; for day 13, n=4 for both groups; for day 21 n=5 (WT) and n=4 (IFN-γKO). Representative of one experiment.

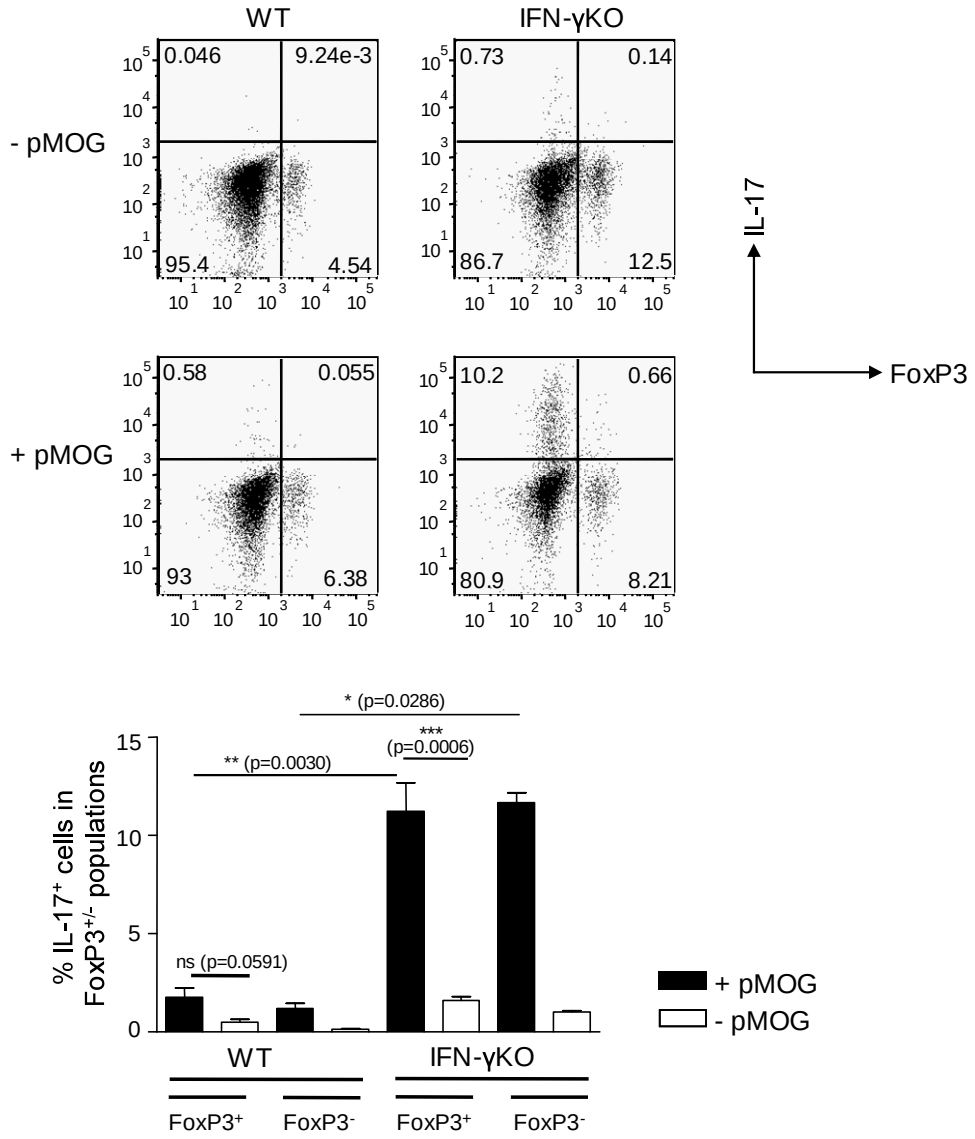

Figure 5.6 Increased production of IL-17 by FoxP3$^+$ Treg in response to pMOG-stimulation in the CNS.

A, gated on CD4$^+$ cells from the CNS of WT (left) and IFN-γKO (right) mice at day 13 post-immunisation (peak of disease in WT mice). Showing IL-17 production by FoxP3$^-$ and FoxP3$^+$ cells after an overnight re-stimulation +/- 20 μg/ml pMOG; **B,** Quantitative representation of IL-17 production, in the presence (black bar) and absence (clear bar) of pMOG stimulation, by the FoxP3$^+$ and FoxP3$^-$ cells as a proportion of their respective populations. Statistical analyses performed with Mann Whitney test ('*' represents: * $p<0.05$; ** $p<0.01$; *** $p<0.001$). n=4 mice for both groups.

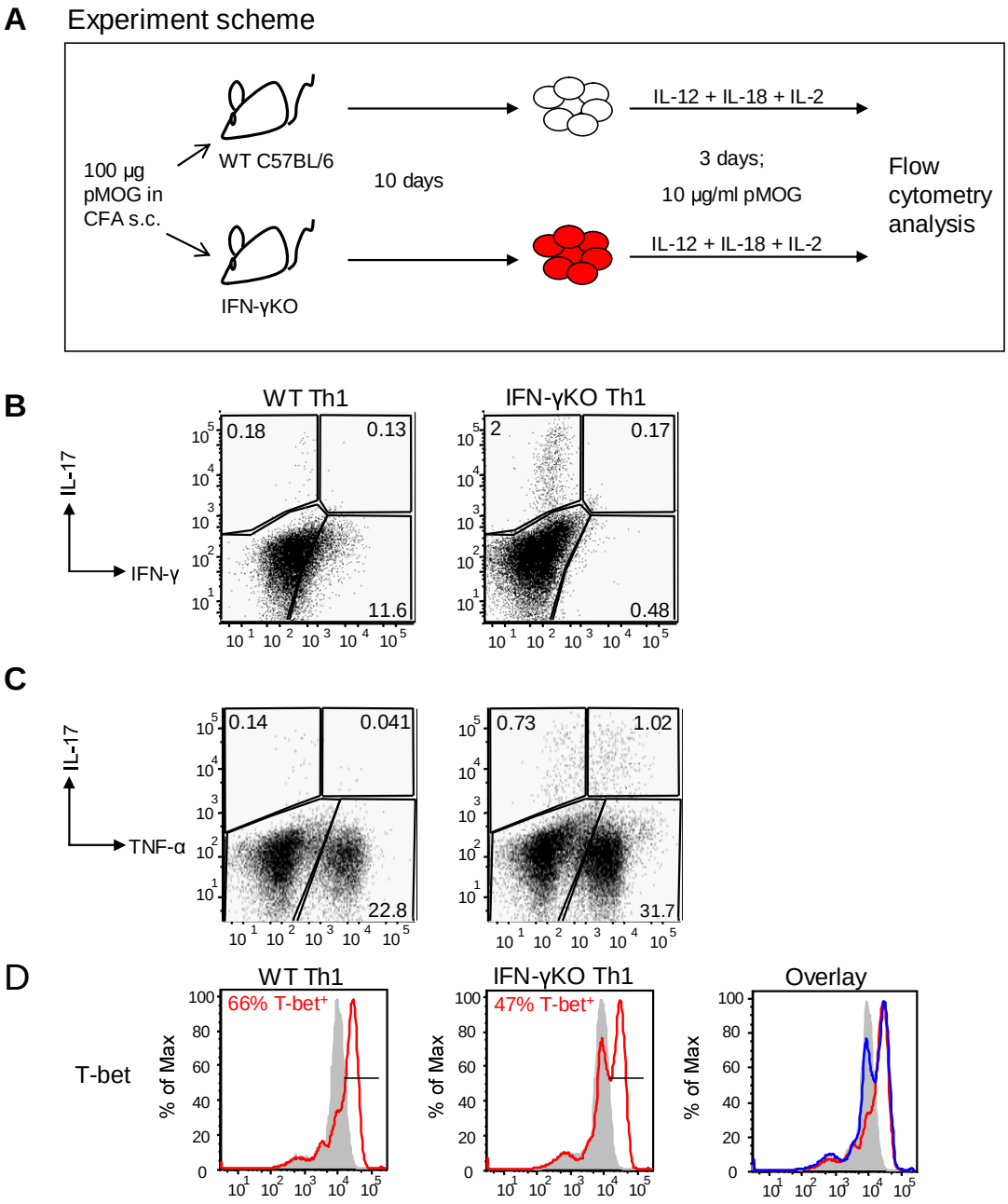

Figure 5.7 Phenotype of *in vitro* polarised pMOG-reactive IFN-γKO Th1 cells.

A, Experiment scheme; gated on $CD4^+$ cells, **B,** IFN-γ and IL-17 production and **C,** TNF-α and IL-17 production by WT Th1 (left) and IFN-γKO Th1 (right) cells after PMA/ionomycin re-stimulation; **D,** gated on $CD4^+$ cells, T-bet expression on WT (**red**, left) and IFN-γKO (**red**, middle) Th1 cells versus the IgG1 isotype control (**grey fill**), and overlay of T-bet expression on WT Th1 (**red**), IFN-γKO Th1 (**blue**) and isotype control (**grey fill**). n=1 for both cell populations. Increased IL-17 production observed in IFN-γKO cells representative of two experiments. The same trend but at much higher levels is observed in Figure 5.8.

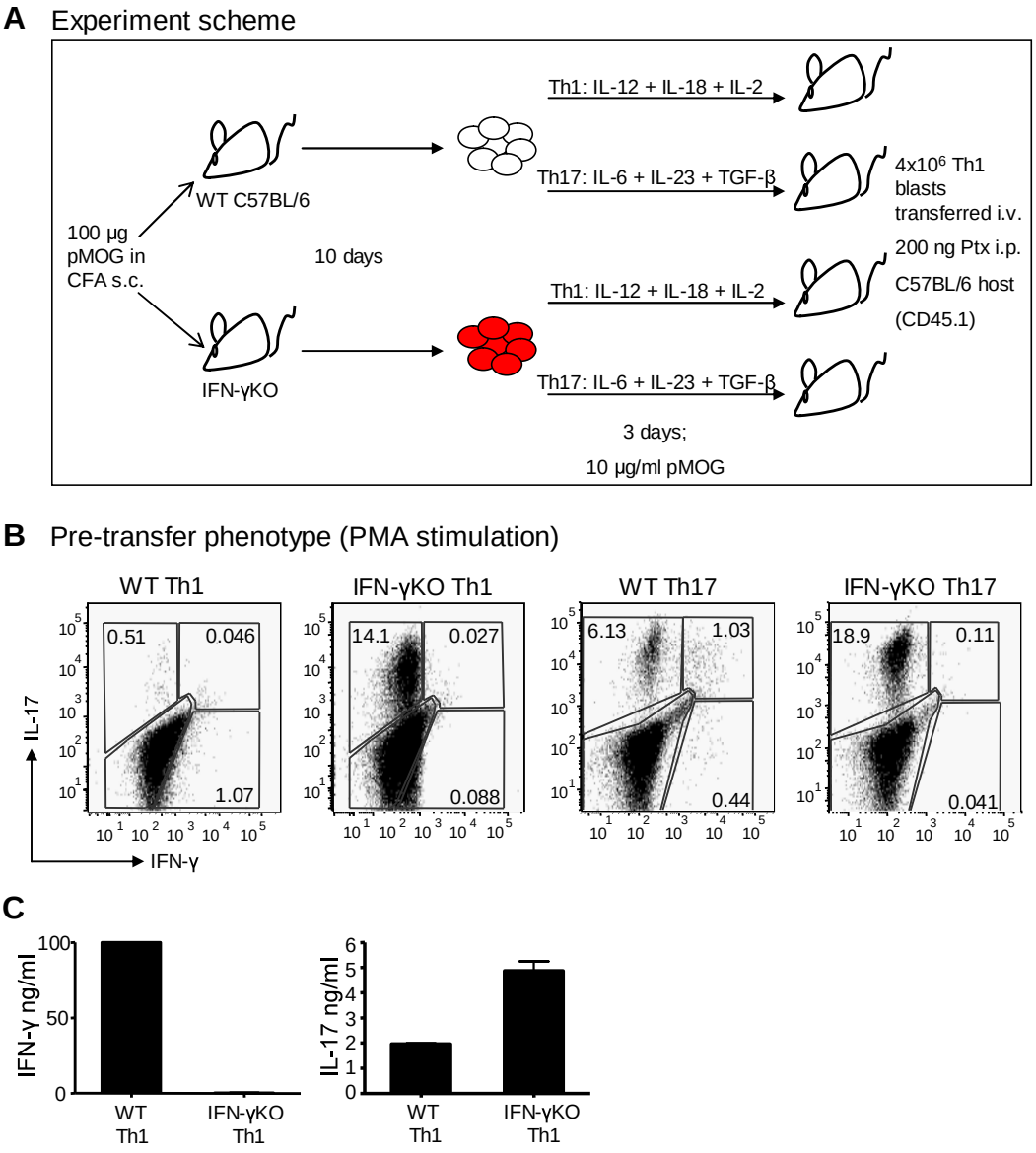

Figure 5.8 Pre-transfer phenotype of pMOG-reactive WT and IFN-γKO Th1 cells.

A, Experiment scheme; **B,** Phenotype of pMOG-reactive WT and IFN-γKO Th1 cells and WT and IFN-γKO Th17 cells after PMA/ionomycin stimulation, gated on $CD4^+$ cells, showing IFN-γ and IL-17 production; **C,** IFN-γ and IL-17 production by WT and IFN-γKO Th1 cells as determined by ELISA using supernatants obtained after 3 days of *in vitro* polarisation of cells. Higher mean fluorescence intensity (MFI) values in Th17 $IL\text{-}17^+$ populations: IFN-γKO Th1 (10039), WT Th17 (25831) and IFN-γKO Th17 (20438).

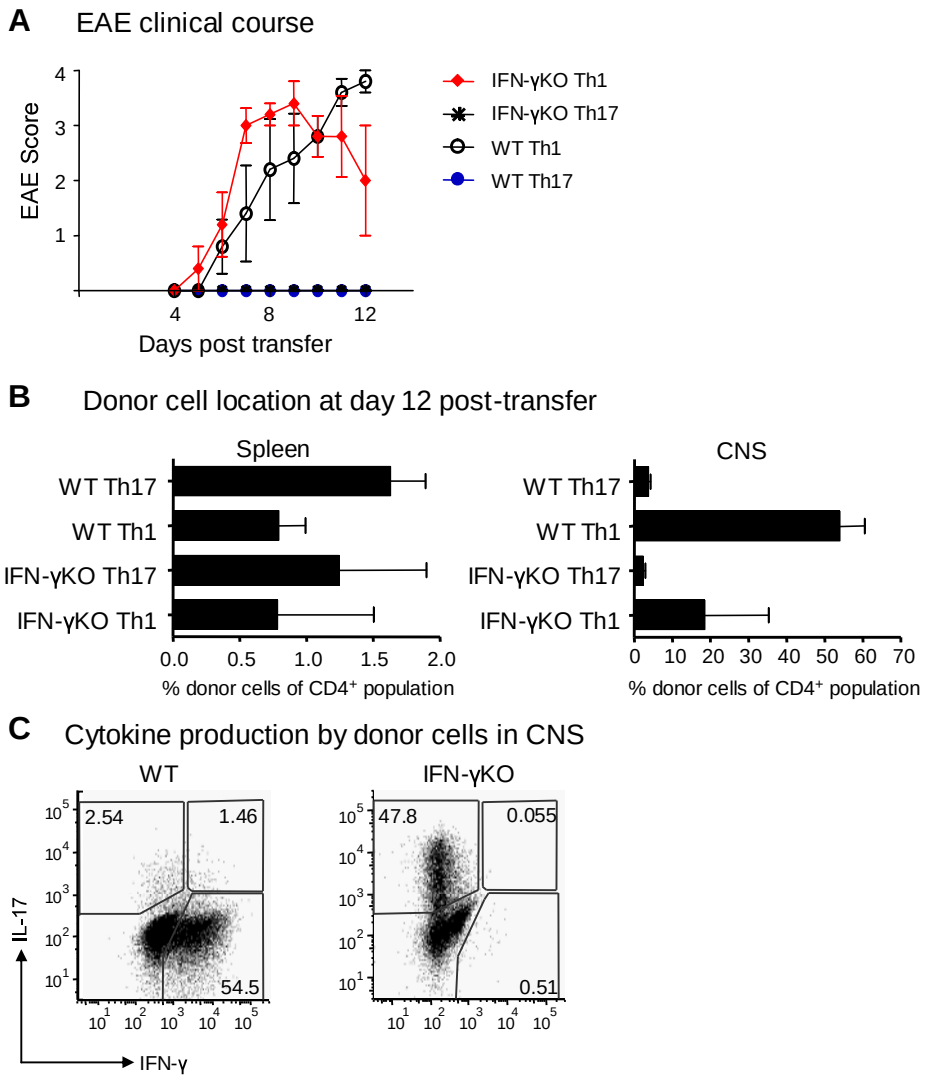

Figure 5.9 pMOG-reactive IFN-γKO Th1 cells are still capable of inducing EAE despite not producing IFN-γ.

A, Mean EAE scores after transfer of WT Th1 (○), WT Th17 (●), IFN-γKO Th1 (♦) and IFN-γKO Th17 (✱) cells, neither Th17 population was able to induce disease; **B,** Percentage donor cell infiltration into the spleen (left) and CNS (right) at day 12 post-transfer; **C,** gated on the donor $CD4^+$ cells, in the CNS at the peak of disease IL-17 and IFN-γ production by the donor WT Th1 cells (left) and the donor IFN-γKO Th1 cells (right), after re-stimulation with PMA/ionomycin (pooled CNS samples from each group). Disease incidence: WT Th1 (5/5), WT Th17 (0/5), IFN-γKO Th1 (5/5) and IFN-γKO Th17 (0/4). Proportion of mice with severe EAE is significantly higher in WT Th1 transfers compared to WT Th17 (p=0.0079) and IFN-γKO Th17 (p=0.0079). No difference in proportion of mice with severe EAE between WT Th1 and IFN-γKO Th1 transfers. See Appendix 11 for summary of p values. Statistics performed using Fisher's exact test. No difference in percentage of donor cells in the spleen (p=0.3916) or CNS (p=0.3916) between the groups, as determined by Kruskal-Wallis test. Representative of two experiments.

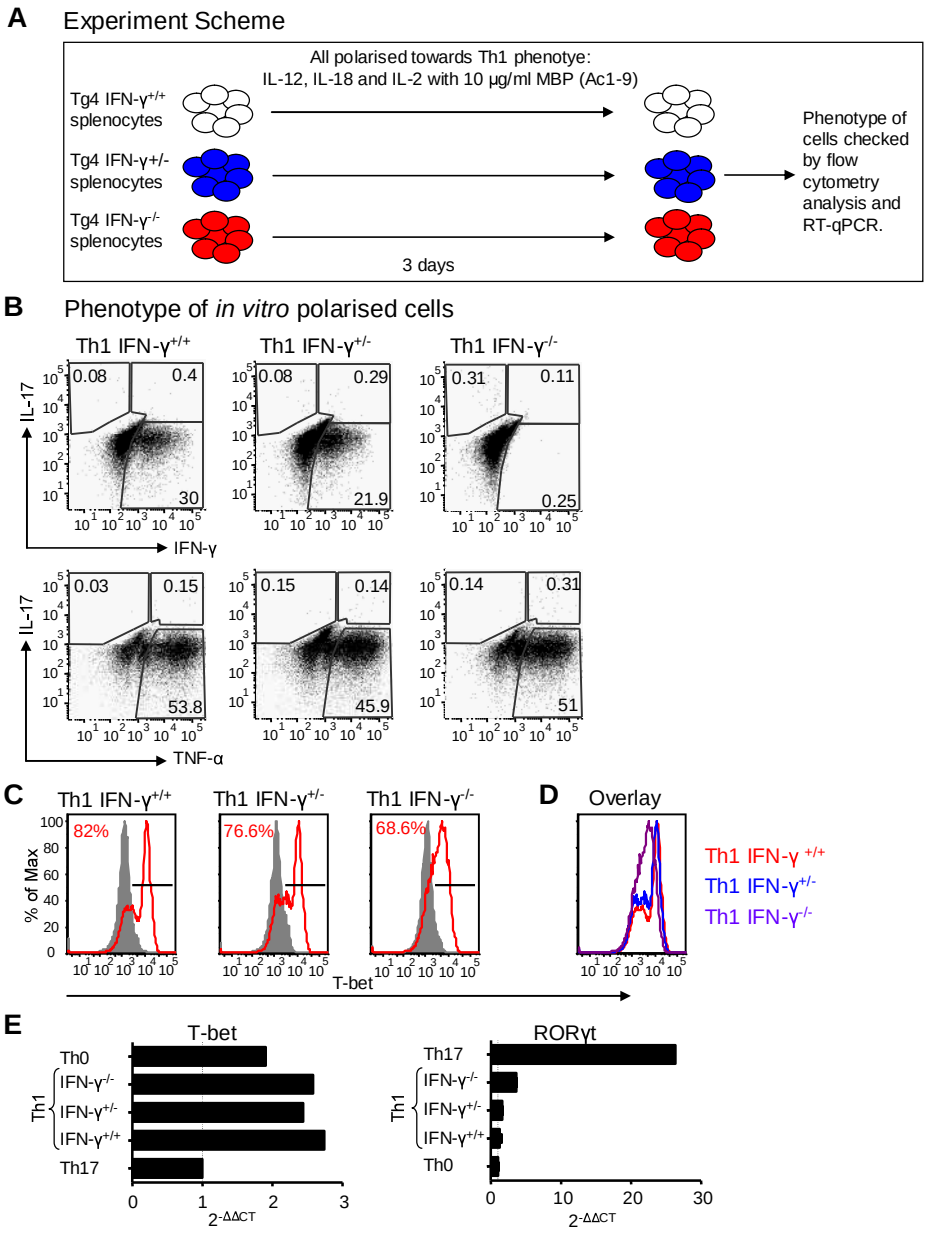

Figure 5.10 Phenotype of TCR transgenic Tg4 Th1 IFN-γ$^{+/+}$, IFN-γ$^{+/-}$ and IFN-γ$^{-/-}$ cells.

A, Experiment scheme; **B,** gated on CD4^{+} cells, IFN-γ, IL-17 and TNF-α production by Tg4 Th1 IFN-γ$^{+/+}$ (left), IFN-γ$^{+/-}$ (middle) and IFN-γ$^{-/-}$ (right) cells; **C,** gated on CD4^{+} cells, T-bet expression on Tg4 Th1 IFN-γ$^{+/+}$, IFN-γ$^{+/-}$ and IFN-γ$^{-/-}$ cells (**red**) versus IgG1 isotype control (**grey fill**); **D,** overlay of T-bet expression on Th1 IFN-γ$^{+/+}$ (**red**), Th1 IFN-γ$^{+/-}$ (**blue**) and Th1 IFN-γ$^{-/-}$ (**purple**) cells highlighting percentage of T-bet+ cells in gate; **E,** RT-qPCR for T-bet (left) and RORγt (right) mRNA expression on Th1 polarised IFN-γ$^{+/+}$, IFN-γ$^{+/-}$ and IFN-γ$^{-/-}$ cells, and Th0 and Th17 cells using Th17 cells as the control for T-bet expression, and Th0 cells as the control for RORγt expression. Dotted line represents control $2^{-\Delta\Delta CT}$=1. Staining representative of three experiments.

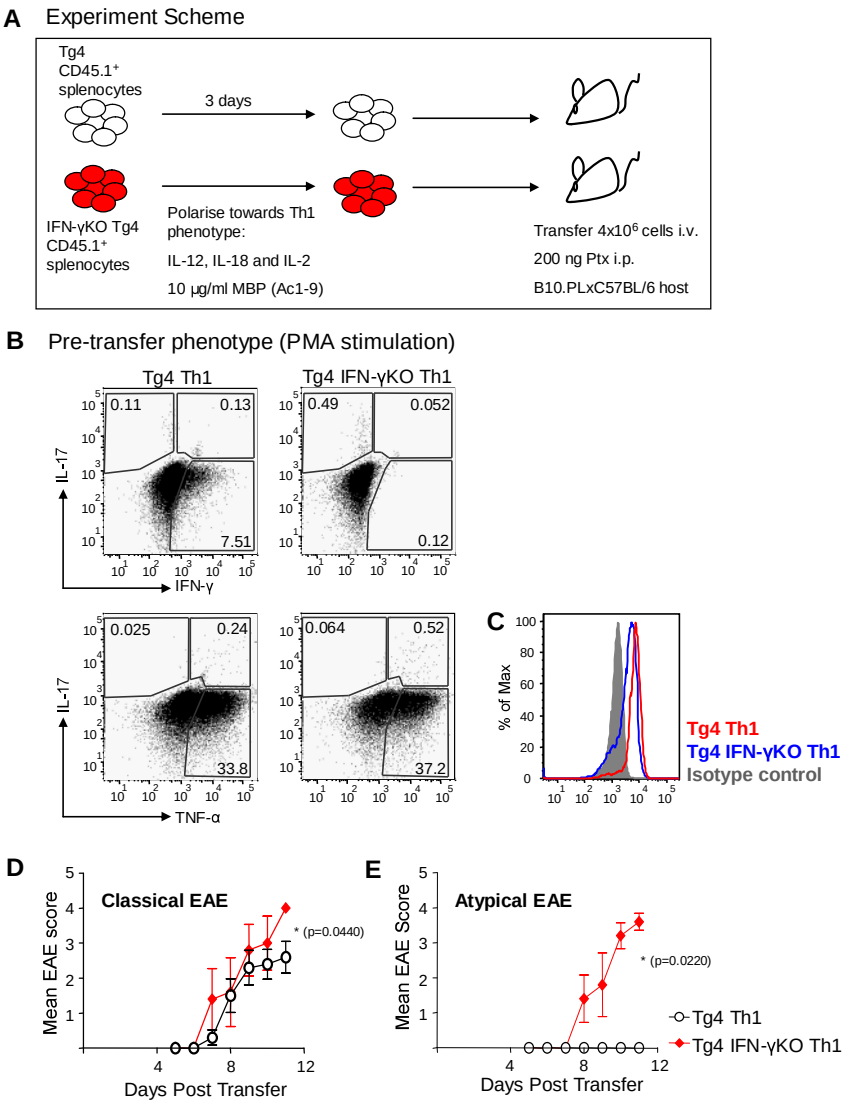

Figure 5.11 TCR transgenic Tg4 IFN-γKO Th1 cells induce both classical and atypical EAE.

A, Experiment scheme; Pre-transfer phenotype of Tg4 WT and IFN-γ KO Th1 cells, gated on $CD4^+$ cells showing **B,** IFN-γ, IL-17 and TNF-α production after PMA/ionomycin re-stimulation; and **C,** T-bet expression on Tg4 WT Th1 (**red**), Tg4 IFN-γKO Th1 (**blue**) and the isotype control (**grey fill**); **D,** Mean EAE scores after transfer of Tg4 WT Th1 (○) and Tg4 IFN-γKO Th1 (♦) cells for both classical (left) and atypical (right) EAE. Error bars represent mean ± standard error. Disease incidence: WT Th1 cells: 10/10 classical EAE, 0/10 atypical EAE; IFN-γKO Th1 cells: 5/10 classical EAE, 5/10 atypical EAE. The classical disease course (D) represents only mice that developed classical disease, and the atypical disease course (E) represents the mice that developed atypical disease in each transfter group. The proportion of mice showing severe classical EAE is significantly higher in the IFN-γKO Th1 transfer; the proportion of mice exhibiting severe atypical EAE is significantly higher in the IFN-γKO transfers compared to the WT Th1 transfers. EAE statistical analyses performed using Fisher's exact test. Representative of three experiments.

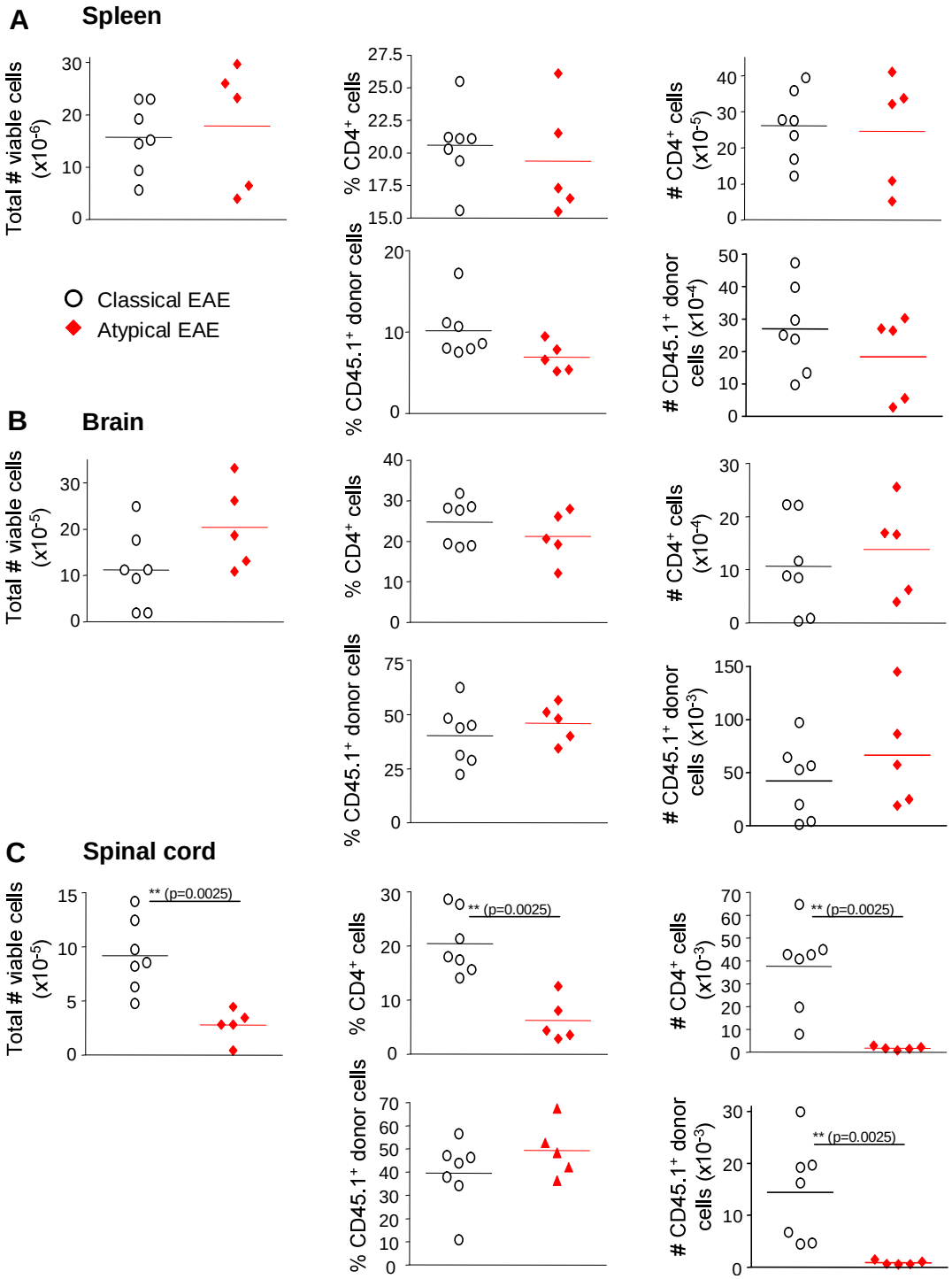

Figure 5.12 Donor cell infiltration into the brain, spinal cord and spleen during classical and atypical EAE.

Total number viable cells, percentage and absolute numbers of $CD4^+$ T cells and $CD45.1^+$ donor cells in the **A,** spleen, **B,** brain; and **C**, spinal cord at day 11 post-transfer in classical (○) versus atypical (♦) EAE. Statistical analyses performed using the Mann Whitney test ('*' represents: * $p<0.05$; ** $p<0.01$; *** $p<0.001$). See Appendix 12 for representative flow cytometry gating strategy. For harvest, n=7 classical, n=5 atypical disease. Representative of two experiments.

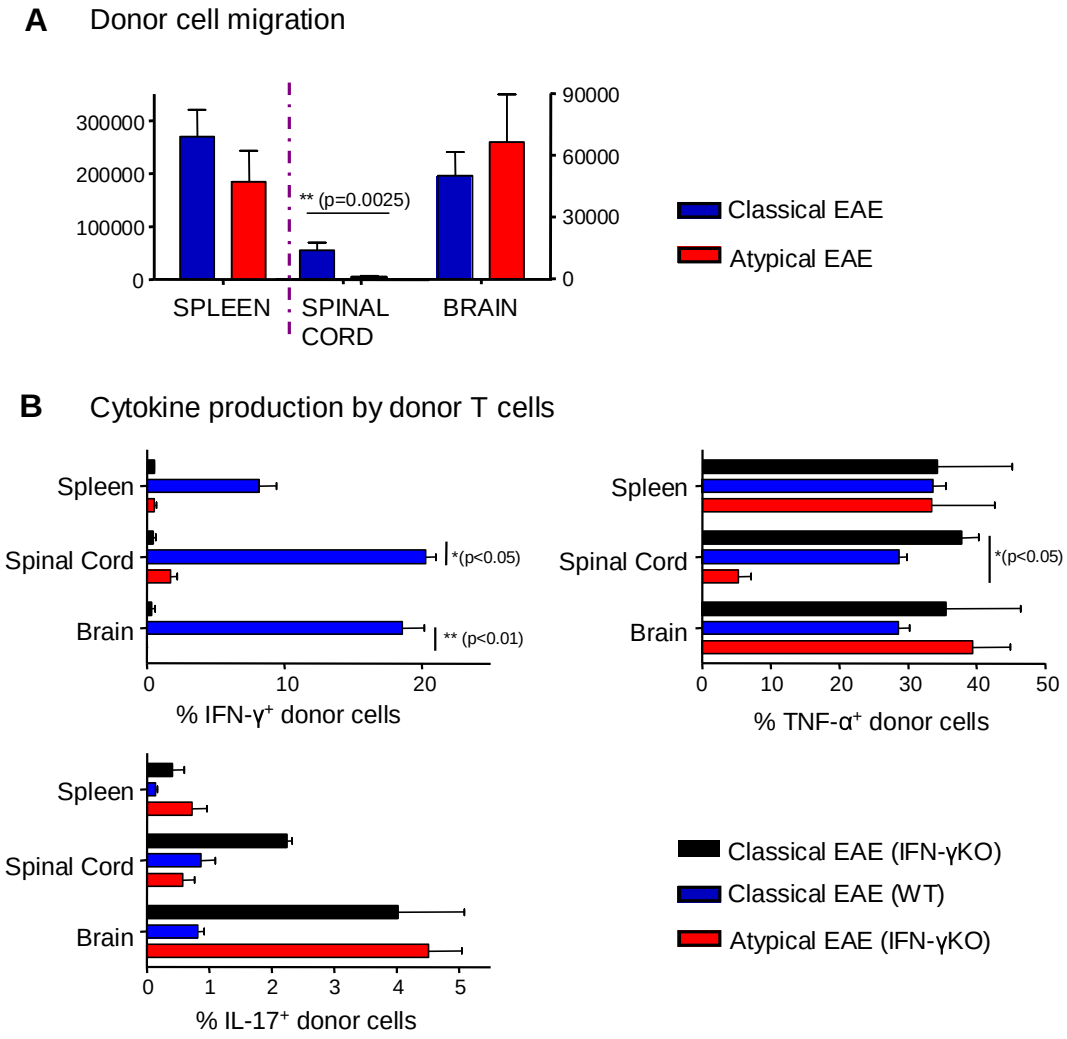

Figure 5.13 Donor cells infiltrate into the brain specifically and not the spinal cord during atypical EAE.

A, donor cell infiltration during classical (**blue**) and atypical (**red**) EAE in the spleen, spinal cord and brain taken at day 11 post-transfer; **B,** IFN-γ, IL-17 and TNF-α production by donor T cells in the spleen, spinal cord and brain during classical (**blue**) and atypical (**red**) EAE. Statistical analyses performed using Kruskal-Wallis test (KW p values: Spinal cord IFN-γ *p=0.0115; Brain IFN-γ **p=0.0088; spinal cord TNF-α **p=0.0090) and Dunn's multiple comparison post test ('*' represents: * p<0.05; ** p<0.01; *** p<0.001). See Appendix 12 B for representative flow cytometry gating strategy for cytokine staining. For harvest, n=7 classical, n=5 atypical. Representative of two experiments.

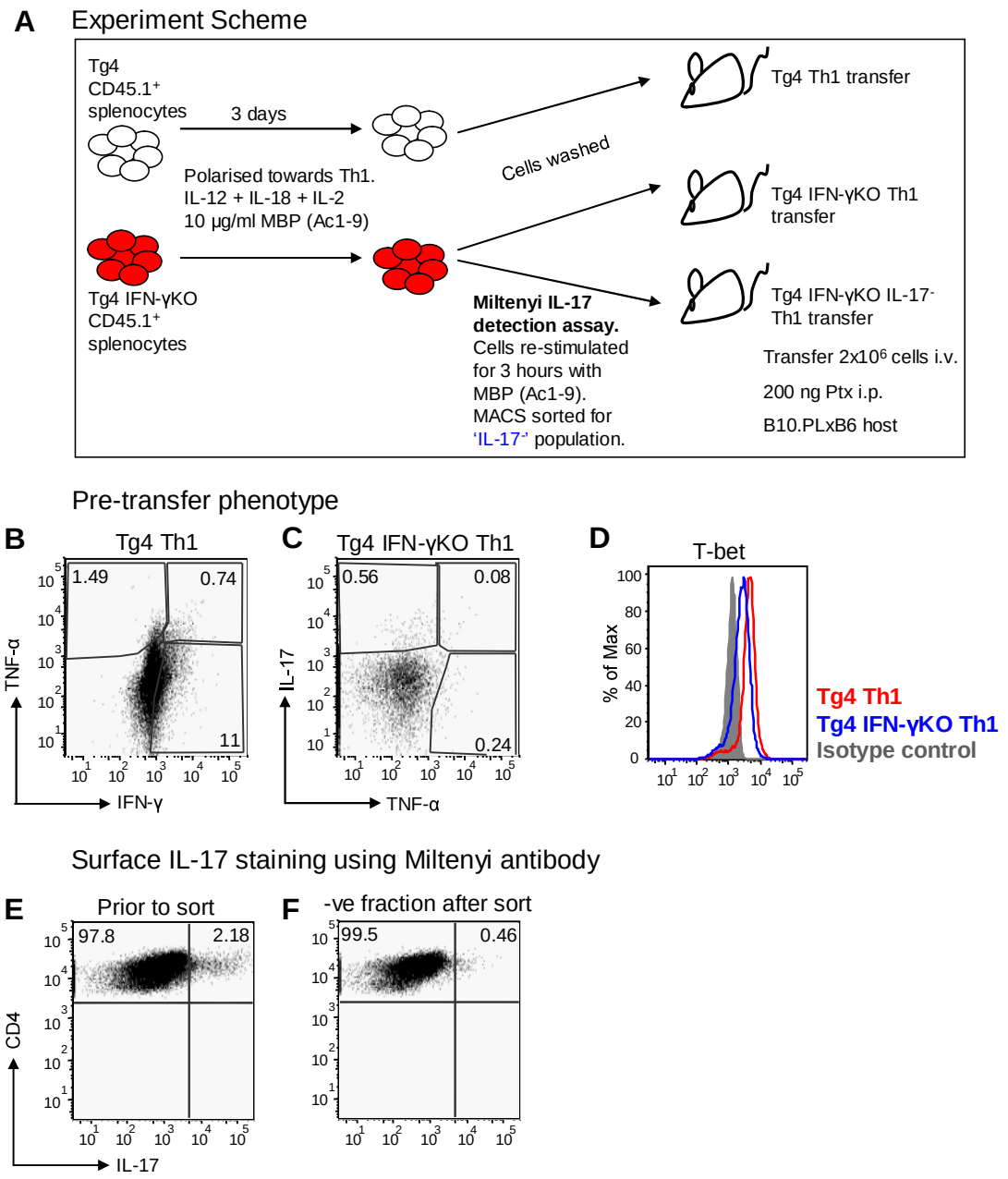

Figure 5.14 Requirements of IL-17 for the induction of atypical EAE.

A, Experiment scheme; Pre-transfer phenotype of transferred gated on $CD4^+$ cells showing **B,** WT Th1 cells (IFN-γ and TNF-α production) and **C,** IFN-γKO Th1 cells (TNF-α and IL-17 production); and **D,** T-bet expression on WT Th1 (**red**), IFN-γKO Th1 (**blue**) and IgG1 isotype control (**grey fill**); and surface staining of IL-17 using 'Miltenyi IL-17 detection and secretion kit' after 3 hours of re-stimulation with MBP (Ac1-9) showing percentage of $IL\text{-}17^+$ cells **E,** prior to; and **F,** after IL-17 MACS sort in the Tg4 IFN-γKO Th1 population.

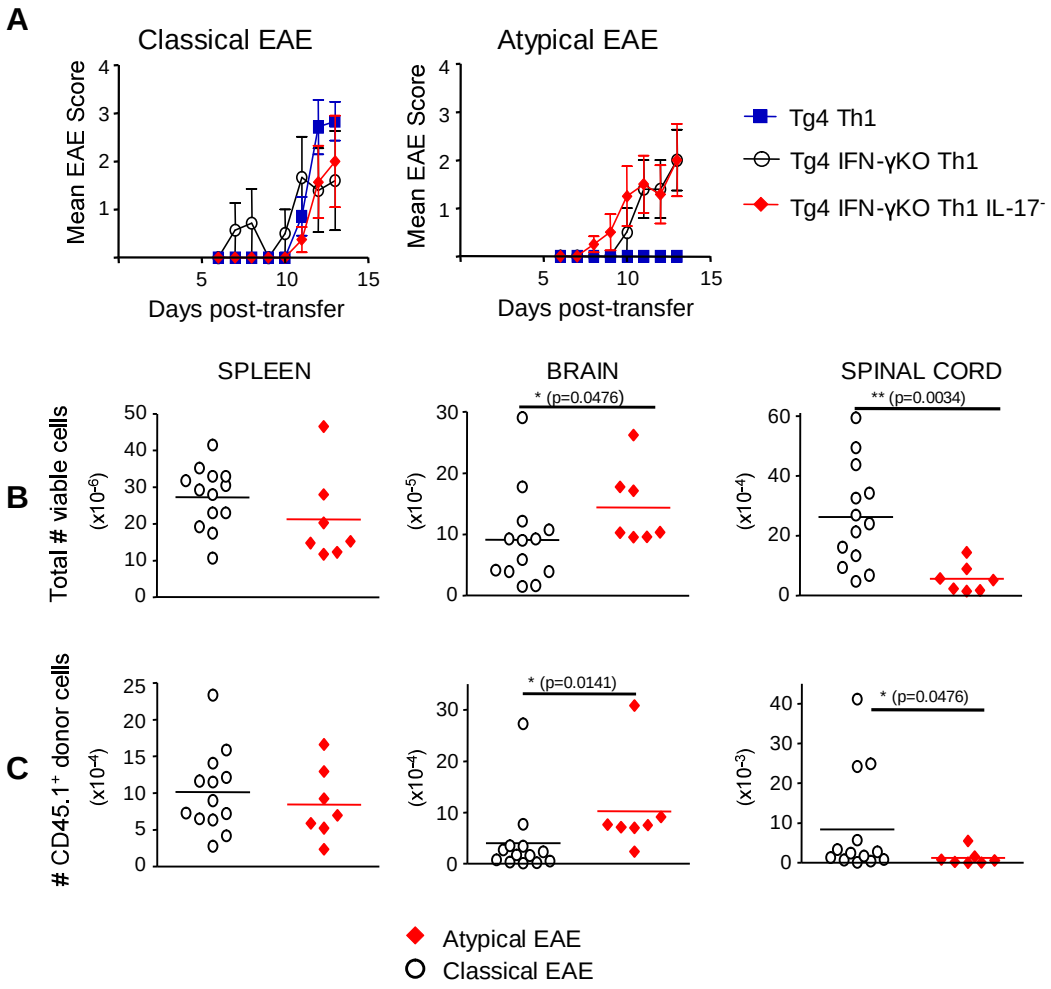

Figure 5.15 Tg4 IFN-γKO IL-17$^-$ Th1 cells are still capable of inducing atypical EAE.

A, Mean EAE scores after transfer of Tg4 Th1 (■), Tg4 IFN-γKO Th1 (○) and Tg4 IFN-γKO IL-17$^-$ Th1 (♦) cells in terms of classical (left) and atypical (right) EAE; Day 13 post-transfer, **B,** total number of viable cells in the spleen (left), brain (middle) and spinal cord (right); **C,** number of CD45.1$^+$ donor cell numbers in the spleen (left), brain (middle) and spinal cord (right). Disease incidence: WT Th1 (7/8): 7/8 classical EAE, 0/8 atypical EAE; IFN-γKO Th1 (8/8) 4/8 classical EAE, 4/8 atypical EAE; IFN-γKO Th1 IL-17-(8/8) 3/8 classical EAE, 5/8 atypical EAE. No significant differences in EAE severity as determined by Fisher's exact test. See Appendix 13 A and B for summary of p values. Error bars represent mean ± standard error. Statistical analyses performed using Mann Whitney test ('*' represents: * p <0.05; ** p <0.01; *** p <0.0001). For harvest, n=13 for classical, n=7 for atypical disease. IL-17 depletion representative of one experiment.

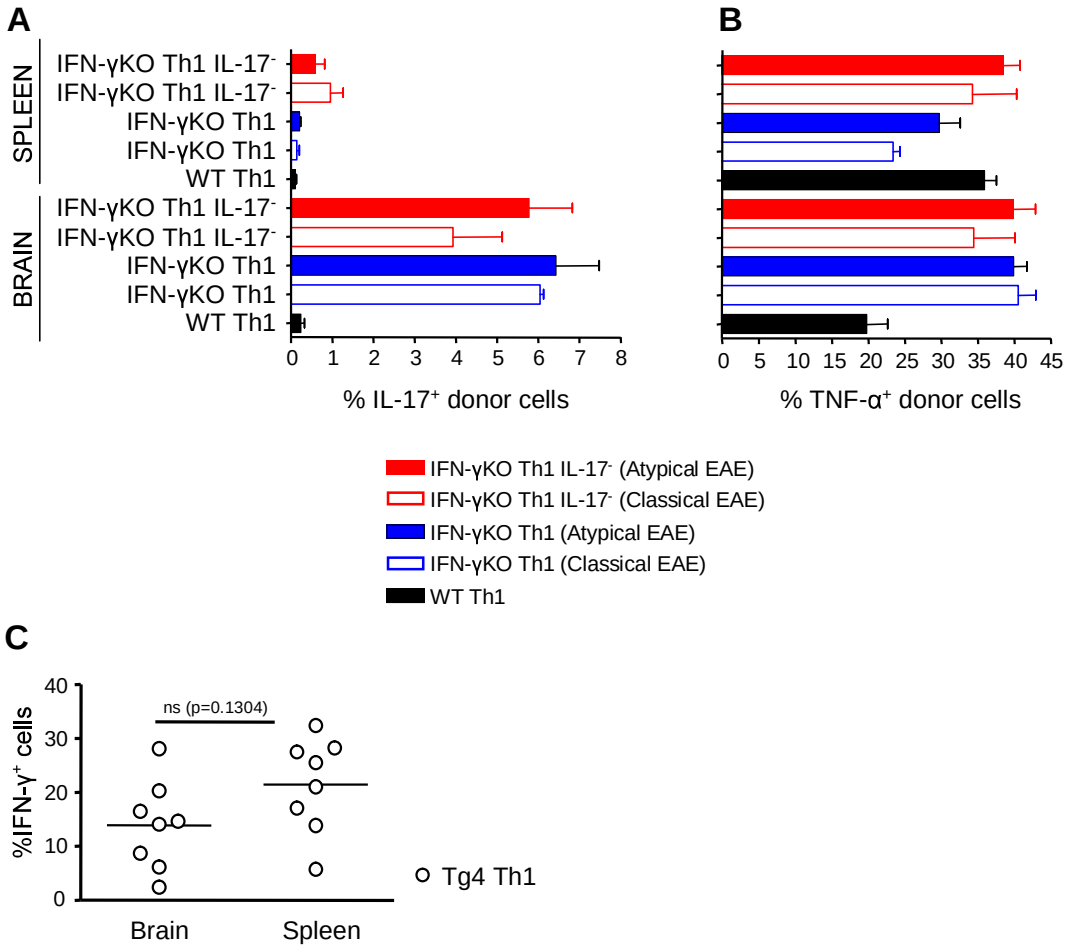

Figure 5.16 Increased production of IL-17 in the brain by Th1 IFN-γKO and Th1 IFN-γKO IL-17$^-$ cells at day 13 post-transfer compared to those in the spleen.

Gated on CD4$^+$ CD45.1$^+$ donor cells, percentage of **A,** IL-17$^+$; **B,** TNF-α$^+$ and **C,** IFN-γ$^+$ cells in the brain and the spleen at day 13 post-transfer after an over-night re-stimulation in the presence of 20 μg/ml MBP (Ac1-9) and four hours of incubation with Brefeldin A. A and B highlight cytokine production by the WT Th1 cells (**black bar**), IFN-γKO Th1 cells (**blue bars**), and IFN-γKO Th1 IL-17$^-$ cells (**red bars**). In the IFN-γKO donor cells, open bars represent the donor cells that gave rise to classical disease, and closed bars represent donor cells that gave rise to atypical EAE. There were significantly higher percentages of IL-17$^+$ cells in the brain of EAE mice with IFN-γKO Th1 and IFN-γKO Th1 IL-17$^-$ cells transferred, compared to the spleen with WT Th1 cells transferred (* $p<0.05$). No significant difference in proportion of TNF-α$^+$ cells or IFN-γ$^+$ cells in the brain and spleen. Statistics performed using the Kruskal-Wallis test (KW p value: ***p=0.0003 for A) and Dunn Multiple comparison post test (A, B) and the Mann Whitney test (C). For harvest, n=13 for classical, n=7 for atypical disease.

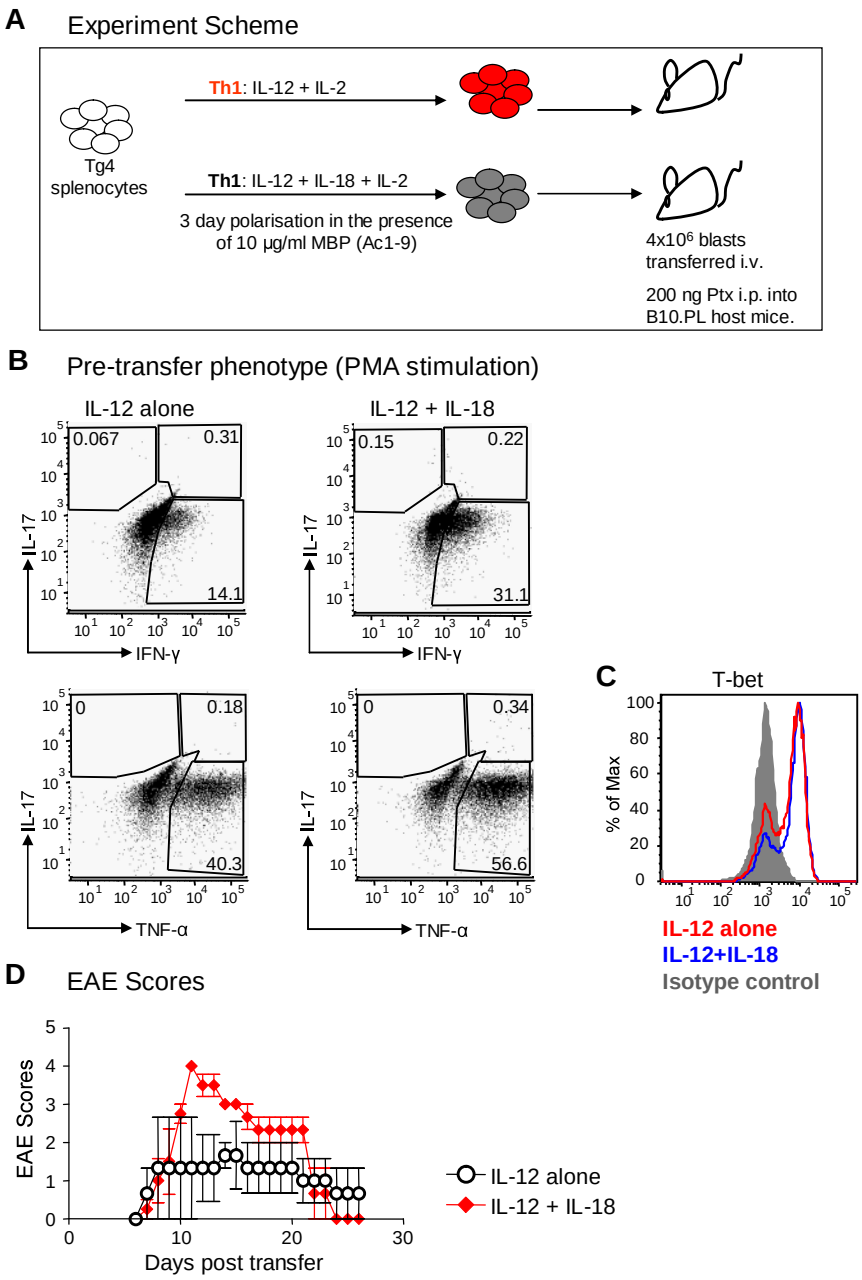

Figure 5.17 Tg4 Th1 polarisation in the presence of IL-18 results in increased IFN-γ and TNF-α production and increased disease incidence and severity.

A, Experiment scheme; **B,** Pre-transfer phenotype of Tg4 Th1 cells polarised with IL-12 alone (left), and IL-12 and IL-18 (right), gated on $CD4^+$ cells, showing IFN-γ and IL-17 production (left) and TNF-α and IL-17 production (right); **C,** gated on $CD4^+$ cells, T-bet expression on IL-12 (**red**), IL-12 and IL-18 (**blue**) Th1 polarised cells and IgG1 isotype control (**grey fill**); **D,** EAE scores after transfer of Tg4 cells polarised in the presence of IL-12 alone (○) or IL-12 and IL-18 (♦). Disease incidence: IL-12 alone (3/4) or IL-12 and IL-18 (4/4). Error bars represent mean ± standard error. The proportion of mice with severe EAE is not significantly higher, as determined by the Fisher's exact test (p=0.1429). Representative of one experiment.

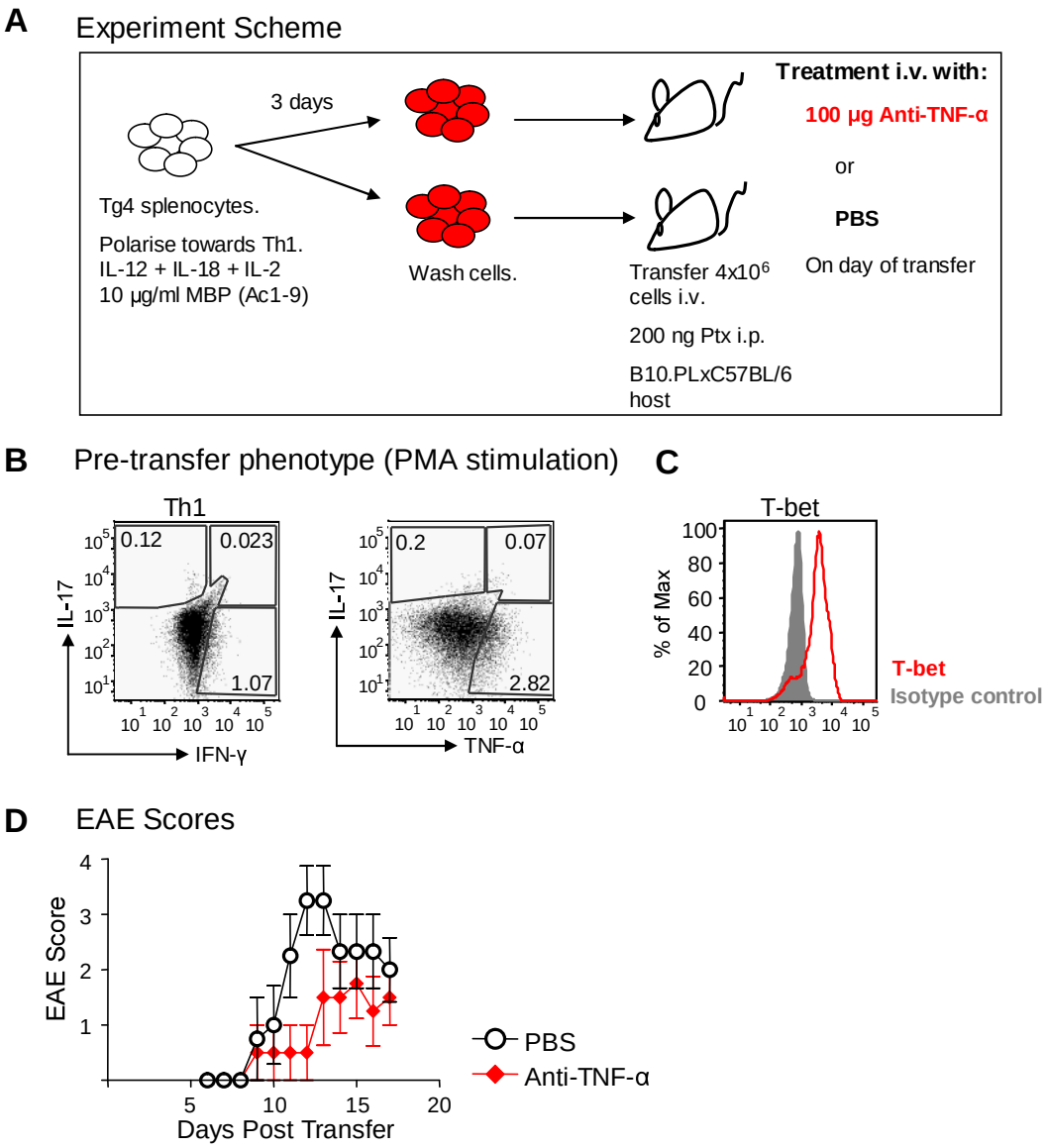

Figure 5.18 Neutralising TNF-α results in decreased severity of EAE by Tg4 Th1 passive transfer.

A, Experiment scheme; Pre-transfer phenotype of donor Tg4 Th1 cells, gated on $CD4^+$ cells, showing **B,** IFN-γ, TNF-α and IL-17 production; and **C,** T-bet expression (**red**) versus IgG1 isotype control (**grey fill**); **D,** Mean EAE scores after passive transfer of Tg4 Th1 cells and one pre-treatment with anti-TNF-α (♦) or PBS (○). Disease incidence: PBS (4/4), Anti-TNF-α (4/4). No significant difference in disease severity (as determined by Fisher's exact test, p=1.0000). Anti-TNF-α treatment representative of one experiment.

6 General Discussion

This thesis has investigated the pathogenic abilities of myelin-reactive Th1 and Th17 cells in EAE. The results can be summarised as follows:

- Myelin reactive Th1 cells were capable of migrating to the CNS and induce EAE, whereas myelin reactive Th17 cells did not. Only when inflammation was already established could the Th17 cells gain access to the CNS and have an effect on EAE severity.
- *In vitro* polarised Th1 cells expressed higher levels of PSGL-1 than Th17 cells. However, the mRNA expression level of C2GnT-I was comparable on both Th1 and Th17 cells, therefore surface PSGL-1 expression on either cell population was functional. The effect of blocking PSGL-1 *in vitro* prior to transfer led to Th1 cells having a decreased ability to migrate in to the CNS, as determined at a pre-clinical time point. Blocking PSGL-1 *in vivo* resulted in the complete abrogation of disease for the length of treatment indicating an important requirement for PSGL-1 in disease induction.
- IFN-γKO mice exhibited prolonged disease compared to the wild-type C57BL/6 mice. This was not due to a decrease in Treg accumulation in the CNS.
- Significantly elevated proportions of $CD4^+$ $IL\text{-}17^+$ cells were observed in the CNS of IFN-γKO mice with active EAE, in the $FoxP3^-$ and $FoxP3^+$ cell populations.
- *In vivo* primed pMOG-reactive Th1 cells deficient in IFN-γ induced classical EAE. Importantly these cells expressed T-bet under *in vitro* Th1 conditions despite the absence of IFN-γ.
- Interestingly *in vitro* primed TCR transgenic Tg4 IFN-γKO Th1 cells, also expressing T-bet, induced atypical as well as classical EAE. The increased ability of these IFN-γ-deficient Th1 cells to produce IL-17 may be important for their ability to induce atypical disease.
- TNF-α plays a role in EAE pathogenesis as decreased disease severity was observed in its absence.

6.1 Th1 versus Th17 in EAE induction: What is required for pathogenesis?

Data presented here clearly show that Th1 cells are able to induce disease both in the presence and absence of IFN-γ-signalling. In our model, as well as in a study of CIA (Janke et al., 2010), Th17 cells are unable to induce inflammation when transferred alone. However, other groups have shown Th17 cells to be pathogenic although the stability of the Th17 cells in these studies is still in question (Bending et al., 2009; Jager et al., 2009; Ghoreschi et al., 2010). These Th17 populations all exhibited a phenotype switch from IL-17-producing on input, to IFN-γ-producing when sampled *ex vivo* and re-stimulated. Therefore, it is not clear which cell phenotype, 'Th1' or 'Th17' induced the disease. In addition, IL-17 has been shown to not be required for the induction of EAE (Haak et al., 2009). Data presented here clearly show that when Th17 cells are polarised *in vitro* from *in vivo* primed pMOG-reactive cells, these cells remain stable *in vivo* and importantly do not induce disease (O'Connor et al., 2008). In contrast, when the Th17 cells are polarised from naïve *in vitro* primed TCR transgenic Tg4 cells, these Th17 cells can induce disease, albeit mild and slightly delayed. However, these Th17 cells exhibited phenotypic plasticity and switched towards an IFN-γ-producing phenotype when recovered from the CNS at the peak of disease (O'Connor et al., 2008). In summary, stable *in vivo* primed Th17 cells cannot induce disease whereas phenotypically unstable Th17 appear able to induce disease, bringing the question as to which cell type is encephalitogenic.

What is it that makes Th1 cells, and not Th17 cells, pathogenic if it is not the signature cytokines that the cells produce? One of the key differences between the cell populations is their expression of T-bet the master regulator of Th1 cells. T-bet remains one of the key factors that is consistently reported to be required for EAE induction in numerous different studies (Lovett-Racke et al., 2004; Nath et al., 2006; Gocke et al., 2007; Abromson-Leeman, Bronson and Dorf, 2009; Yang et al., 2009). Disrupting T-bet signalling, either through the use of T-bet deficient mice (Nath et al., 2006) or siRNA against T-bet (Lovett-Racke et al., 2004) resulted in resistance to EAE induction. In addition, T-bet expression on Th1 or Th17 cells has been shown to be required for their encephalitogenicity (Abromson-Leeman, Bronson and Dorf, 2009; Yang et al., 2009). Data presented here would correlate with the requirement

for T-bet expression on encephalitogenic T cells. Th1 cells, both wild-type and IFN-γKO, express T-bet, and these cells induce EAE whereas Th17 cells, which are T-bet$^-$, do not induce disease. STAT1, another Th1-promoting transcription factor is, however, dispensable for EAE induction as STAT1KO mice display enhanced EAE (Bettelli et al., 2004). The loss of STAT4 results in the abrogation of disease induction, with the isoform STAT4β having more of an influence compared to the STAT4α isoform (Chitnis et al., 2001; Mo et al., 2008). Therefore, two transcription factors vital for the generation of a Th1 response, T-bet and STAT4, remain key factors that are consistently required for induction of disease. Perhaps more emphasis should be put on the role of these two molecules in their requirements for Th1/Th17 mediated pathology.

The EAE experiments presented in this thesis always used Ptx for the induction of disease. In our passive transfer models of EAE, Ptx is required for disease induction otherwise the disease induced is of low severity and low incidence. Other groups, have reported that the use of Ptx can inhibit the induction of disease by Th17 cells (Jager et al., 2009). In those studies, Ptx was thought to inhibit the migration of encephalitogenic T cells across the BBB by affecting the chemokine receptor expression on the T cells. However, as Th1 cells were still able to induce disease in the presence of Ptx in our model, this would argue that the Ptx is not affecting the chemokine receptor expression on the Th17 cells to inhibit their migration to the CNS. It has been suggested that Ptx specifically affects the chemokine receptor signalling on Th17 cells, and not Th1 cells (Jager et al., 2009). However, it is debatable whether Ptx would specifically target particular chemokine receptors, especially as it was found that Ptx had no effect on CCR6 expression on Th17 cells *in vitro* (Jager et al., 2009). EAE induction by Th17 cells with the addition of Ptx has been shown to be feasible (McGeachy et al., 2007), supporting the view that Ptx does not specifically affect the migration of Th17 cells to the CNS.

TNF-α was shown to play a role in disease pathogenesis in EAE (Ruddle et al., 1990; Selmaj, Raine and Cross, 1991), and as shown in Chapter 5, although there are also contradictory data suggesting TNF-α is an anti-inflammatory cytokine in EAE (Frei

et al., 1997; Liu et al., 1998). When translated to human autoimmune disease, anti-TNF-α therapy was found to be an effective treatment for RA and Crohn's disease (Moreland et al., 1997; Targan et al., 1997), but it led to the exacerbation of disease in MS patients (van Oosten et al., 1996; Lenercept-MS-Study, 1999). The differential roles of TNF receptors TNFRI and TNFRII (Suvannavejh et al., 2000) may account for the exacerbation of disease in MS as anti-TNF therapeutics, infliximab and etanercept, block the TNFRII signalling (Franklin, 1999; Agnholt and Kaltoft, 2001) which is thought to have a role in the regulation of inflammation in the CNS, rather than its initiation.

Currently there is a drive towards focusing on Th17 cells and their role in inducing CNS autoimmune disease, despite clear evidence that Th1 cells also induce CNS inflammation. Although neither IFN-γ nor IL-17 is required for disease pathogenesis (Ferber et al., 1996; Wensky et al., 2005; Haak et al., 2009), T-bet and STAT4 however remain key in the pathogenesis of CNS autoimmune disease and perhaps research should focus more on other functions of Th1 and Th17 cells that could induce disease rather than on their end signature products.

6.2 Migration of encephalitogenic T cells into the CNS

One of the treatments for MS is Natalizumab, a monoclonal antibody against α4-integrin. α4β1 is fairly widely expressed on all leukocytes, therefore Natalizumab blocks all leukocyte entry in to the CNS. A major concern over the use of Natalizumab as a treatment, comes from the development of PML, a serious demyelinating disease of the CNS (Kleinschmidt-DeMasters and Tyler, 2005; Langer-Gould et al., 2005). This seems to reflect reduced surveillance allowing reactivation of latent JC virus (Engelhardt and Kappos, 2008). The risk of developing PML increases after 2 years of treatment with Natalizumab, so this is now avoided if possible and it is chiefly prescribed to MS patients that are non-responders to IFN-β or glatiramer acetate, and only to new MS patients if they present with unusually high disease activity. The non-specificity of the treatment, blocking all leukocytes from entering the CNS and other tissues was part of the rationale for investigating what molecules influenced the migration of pathogenic Th1 cells into the CNS.

Identification of a more specific therapeutic target that only blocks the entry of the inflammatory encephalitogenic T cells in to the CNS, might allow for normal immune surveillance.

As described in Chapter 4, blockade of PSGL-1 could affect the entry of the pioneer encephalitogenic T cells into the CNS. However, the expression of PSGL-1, like α4β1, is fairly ubiquitous, and therefore blocking PSGL-1 *in vivo* would affect various different cell types, as seen by it affecting the proportion and number of $CD11b^+$ cells in the spleen. Therefore, the likelihood of PSGL-1-blockade being a feasible therapy for MS seems low. It is nonetheless important to determine the role that PSGL-1 has in the entry of pathogenic T cells into the CNS. It has been shown that T-bet is required for the trafficking of Th1 cells to inflammatory sites and has a role in controlling PSGL-1-mediated migration (Lord et al., 2005). It would be interesting to determine whether T-bet-deficient Th1 cells have an impaired ability to migrate to the CNS to induce EAE in our TCR transgenic passive transfer model.

6.3 Classical versus atypical EAE

Interestingly, TCR transgenic IFN-γKO Th1 cells were able to induce both classical and atypical EAE, whereas Tg4 Th1 cells induced only classical EAE. It is not fully understood where encephalitogenic T cells initially enter the CNS, except possibly through the choroid plexus through CCR6/CCL20 signalling (Reboldi et al., 2009). A possible model for the initiation of classical versus atypical EAE is suggested here on Figure 6.1. For the induction of classical disease, Tg4 Th1 cells enter the CNS at the brain-stem and then migrate towards the spinal cord where inflammation is established. In contrast, in atypical disease, the donor IFN-γKO Th1 cells migrate towards the cerebellum and brain-stem and induce local inflammation in this area. The lack of IFN-γ signalling when IFN-γKO Th1 cells were transferred, or the upregulated levels of IL-17 produced by these cells, appeared to localise these donor cells to the brain area, and prevent them from migrating towards the spinal cord, as highlighted in Figure 6.1. This localisation of encephalitogenic T cells to the brain-stem/cerebellum may be mediated by lack of specific chemokine signalling in the absence of IFN-γ production, specifically by the transferred T cells.

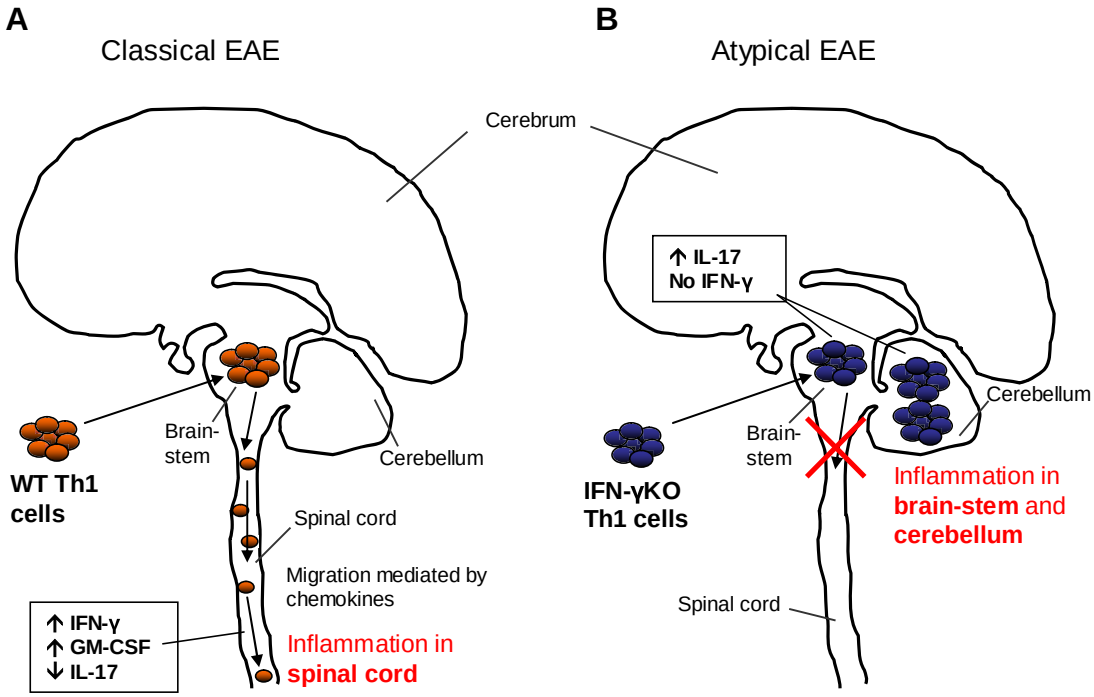

Figure 6.1 Model of migration of TCR transgenic Th1 cells into the CNS to induce classical versus atypical EAE

A, Induction of classical EAE by transfer of WT Th1 cells. The cells enter the CNS via the brain-stem and then migrate down towards the base of the spinal cord, mediated by chemokine gradients. Cells then induce inflammation localised in the spinal cord. **B,** Induction of atypical EAE by transfer of IFN-γKO Th1 cells. These cells enter the CNS via the brain-stem as well, and migrate to the cerebellum as well. Due to the absence of IFN-γ-signalling in the donor cells, increased IL-17 signalling or perhaps the absence of particular chemokine gradients, the cells remain in the brain-stem and cerebellum and induce localised inflammation in that area.

6.4 Future Work

6.4.1 Role of Th17 cells in EAE exacerbation

It would be interesting to determine what role the Th17 cells have in exacerbating disease once they have gained access to the CNS (via the production of IL-17 or another effector function). If the Th1 cells co-transferred with Th17 cells are specifically deleted from the host mice, would the mice recover, or would those Th17 cells that have reached the CNS be able to sustain EAE by themselves? A system that

allows deletion of Th1 or Th17 cells at different time-points would reveal the requirements of each cell type at different stages of disease.

6.4.2 Requirements of IL-17 in induction of atypical EAE

It is important to determine whether IL-17 signalling is required for atypical EAE induction. The use of a blocking antibody against IL-17A would determine whether IFN-γKO Th1 cells can still induce atypical disease in the absence of IL-17-signalling. IL-17FKO mice could also be used to look at the role of pathogenic IFN-γKO Th1 cells in the absence of both IL-17F and IFN-γ-production. Unfortunately this could not be investigated within the timescale of this PhD project.

6.4.3 Is the increased severity of disease observed in IFN-γKO mice attributed to the upregulated IL-17 production?

Increased percentages of IL-17^+ cells were observed in the $FoxP3^-$ and $FoxP3^+$ cells in the CNS of IFN-γKO EAE mice. It would be interesting to determine whether blocking this IL-17 production would result in decreased severity or duration of disease.

6.5 Closing Statement

In summary, this thesis provides data that counter the new dogma that views EAE as a Th17-mediated disease. Th1 cells were able to home to the CNS and induce EAE, whereas Th17 cells did not. PSGL-1 was found to have a role in the entry of the pioneer Th1 cells across the non-inflamed BBB. The project has highlighted that neither 'Th1' nor 'Th17' appears to be an unambiguous descriptor of the T cells that drive pathology. Rather, EAE is a $CD4^+$ T cell-mediated disease with no essential requirement for either IFN-γ or IL-17. In contrast, TNF-α, which can be produced by both Th1 and Th17 cells, does appear to have a role to play in EAE pathogenesis. This thesis has also shown that classical and atypical EAE correlated with inflammation in distinct areas of the CNS (the spinal cord and brain, respectively), which appeared to be differentially sensitive to cytokine signalling.

7 References

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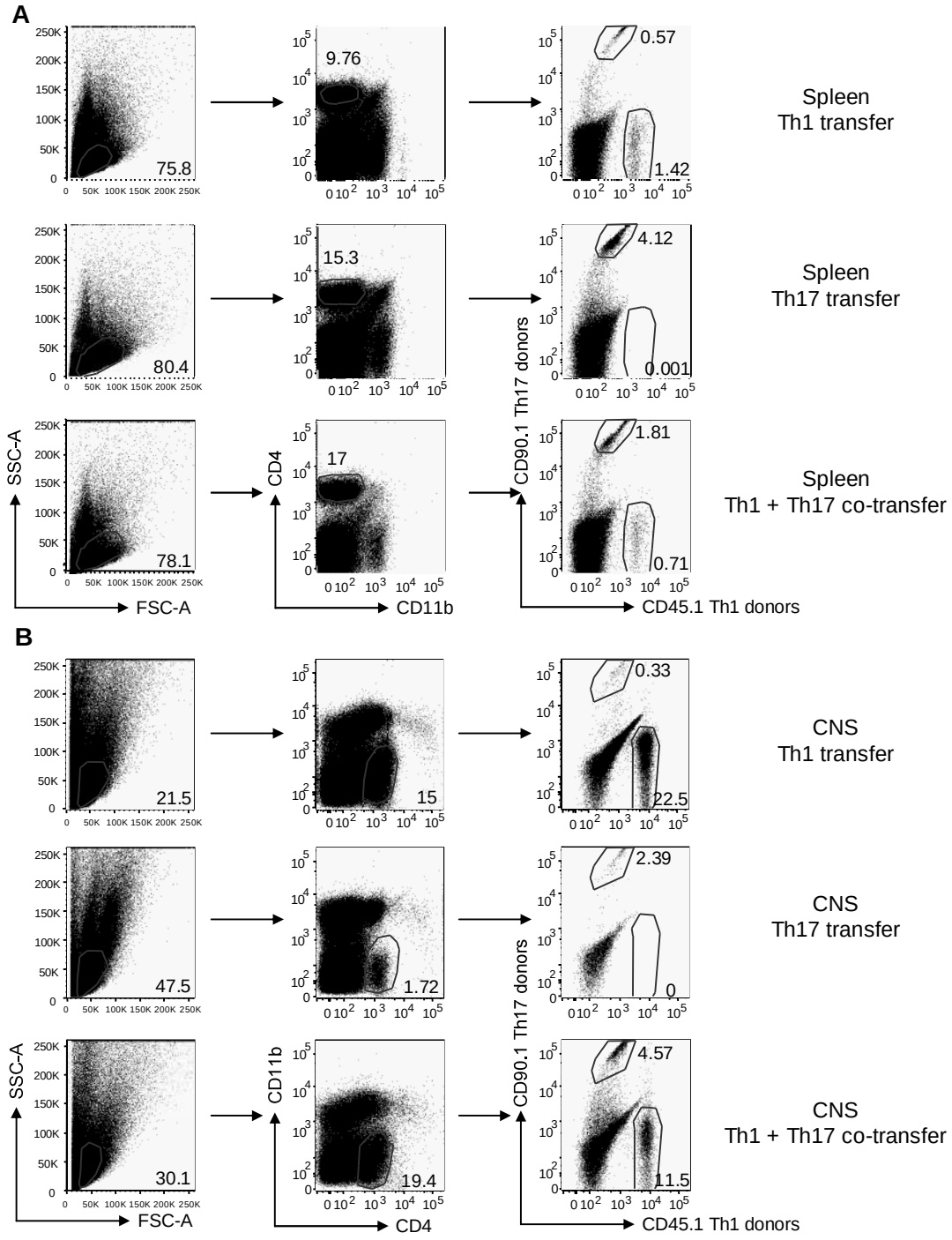
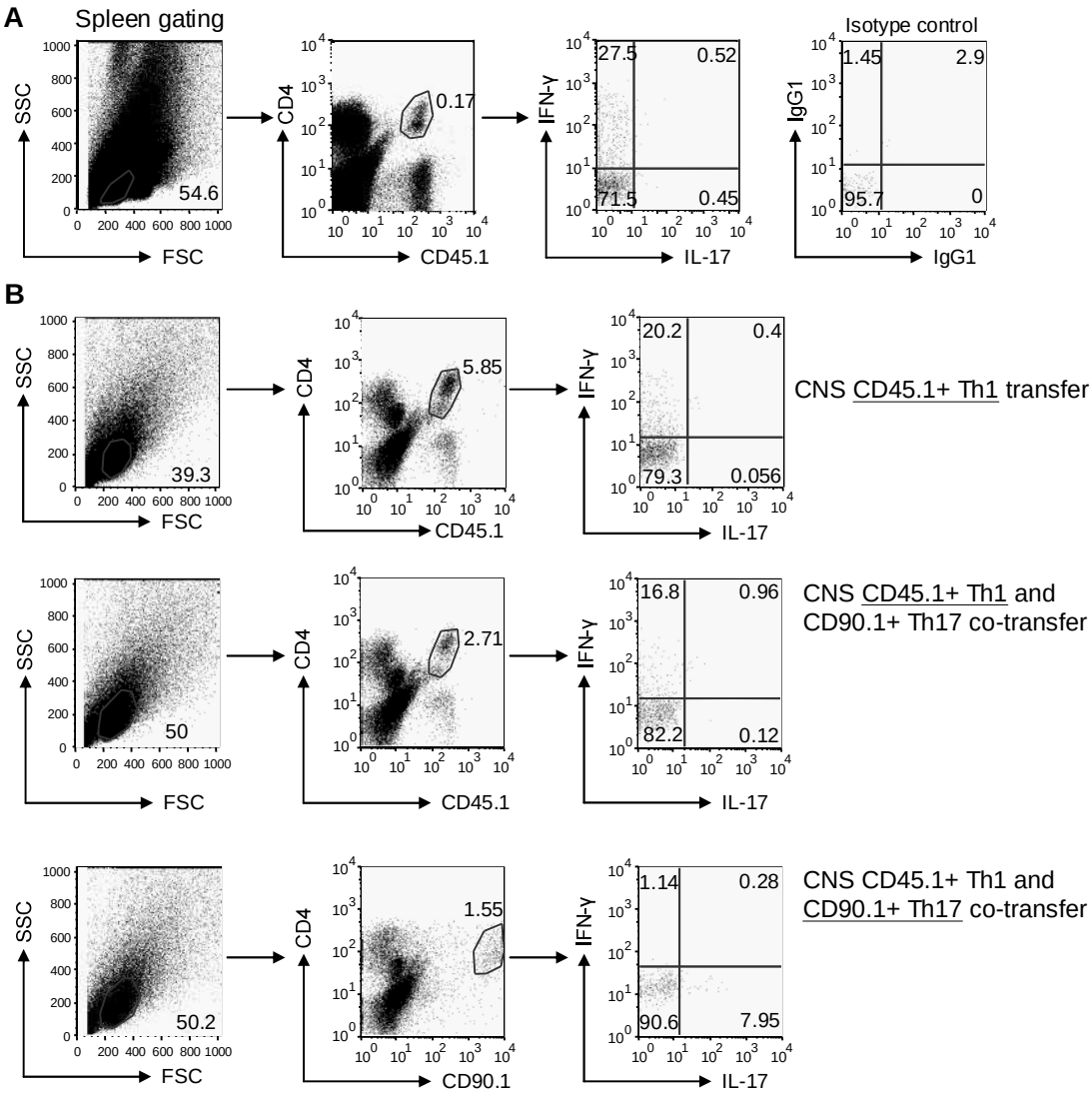
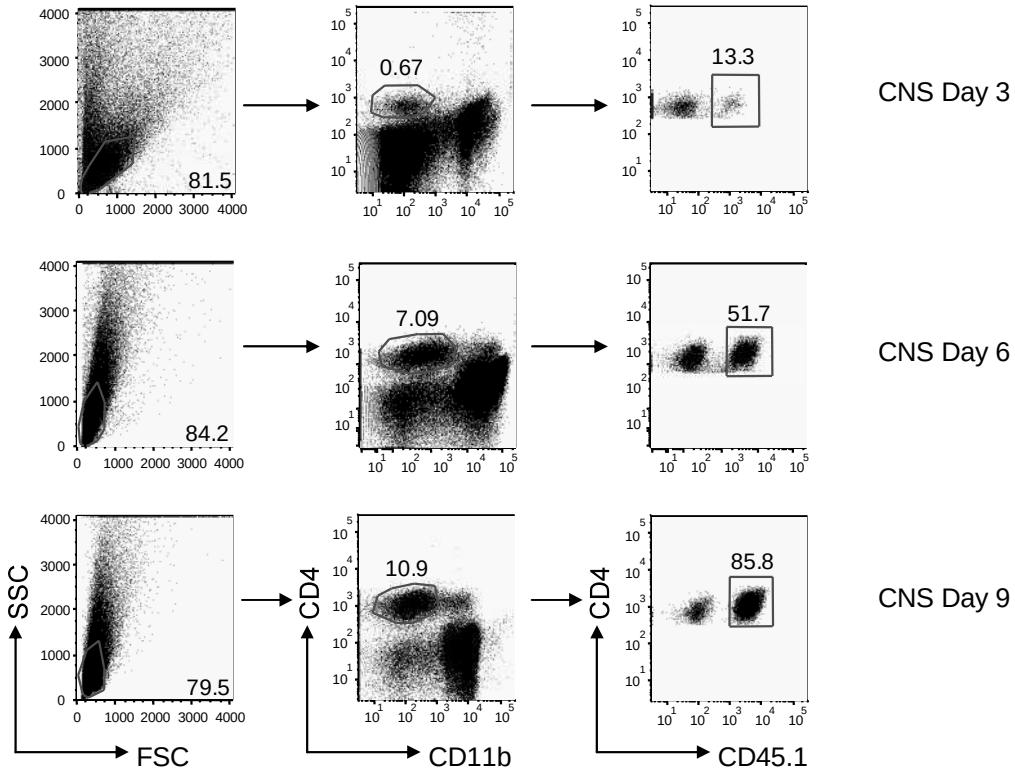
8 Appendix



Appendix 1 Flow cytometry gating strategy for location of donor T cells from Figure 3.4. CD45.1$^+$ Th1 donor cells and CD90.1$^+$ Th17 donor cells in the A, spleen and B, CNS, highlighting the gating strategy. Representative of all gating.



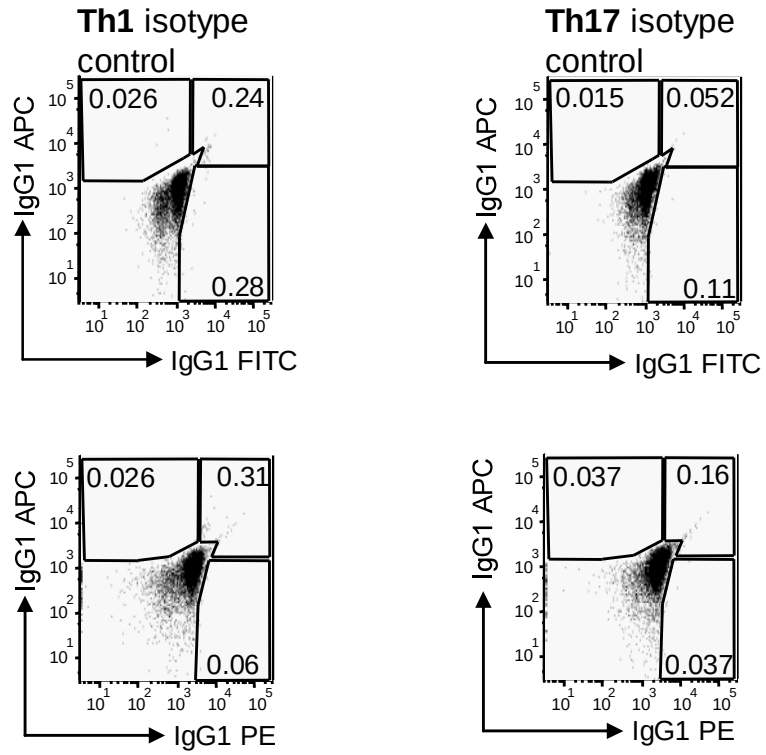
Appendix 2 Flow cytometry gating strategy for donor cell location and cytokine production for Figure 3.6. CD45.1$^+$ Th1 donor cells and CD90.1$^+$ Th17 donor cells cytokine production for IFN-γ and IL-17 in the A, spleen and B, CNS. Representative of gating for all samples.



Appendix 3 Flow cytometry gating strategy for location of donor Th1 cells in the CNS for Figure 3.8. Gating of $CD4^+$ $CD45.1^+$ Th1 donor cells in the CNS at day 3, 6, and 9 post-transfer.

Parameters: # Th1 cells		Significance	p value
$4x10^6$	$2x10^6$	ns	p=1.0000
$4x10^6$	$1x10^6$	ns	p=1.0000
$4x10^6$	$0.5x10^6$	ns	p=0.2500
$4x10^6$	$0.2x10^6$	ns	p=0.2500
$2x10^6$	$1x10^6$	ns	p=1.0000
$2x10^6$	$0.5x10^6$	ns	p=0.4000
$2x10^6$	$0.2x10^6$	ns	p=0.4000
$1x10^6$	$0.5x10^6$	ns	p=1.0000
$1x10^6$	$0.2x10^6$	ns	p=1.0000
$0.5x10^6$	$0.2x10^6$	ns	Not possible

Appendix 4 Fisher's exact test for significant differences in EAE severity for Figure 3.10 A. As determined by comparing number of mice with EAE maximum score of 2 and below, to EAE mice with a score 3 and above.

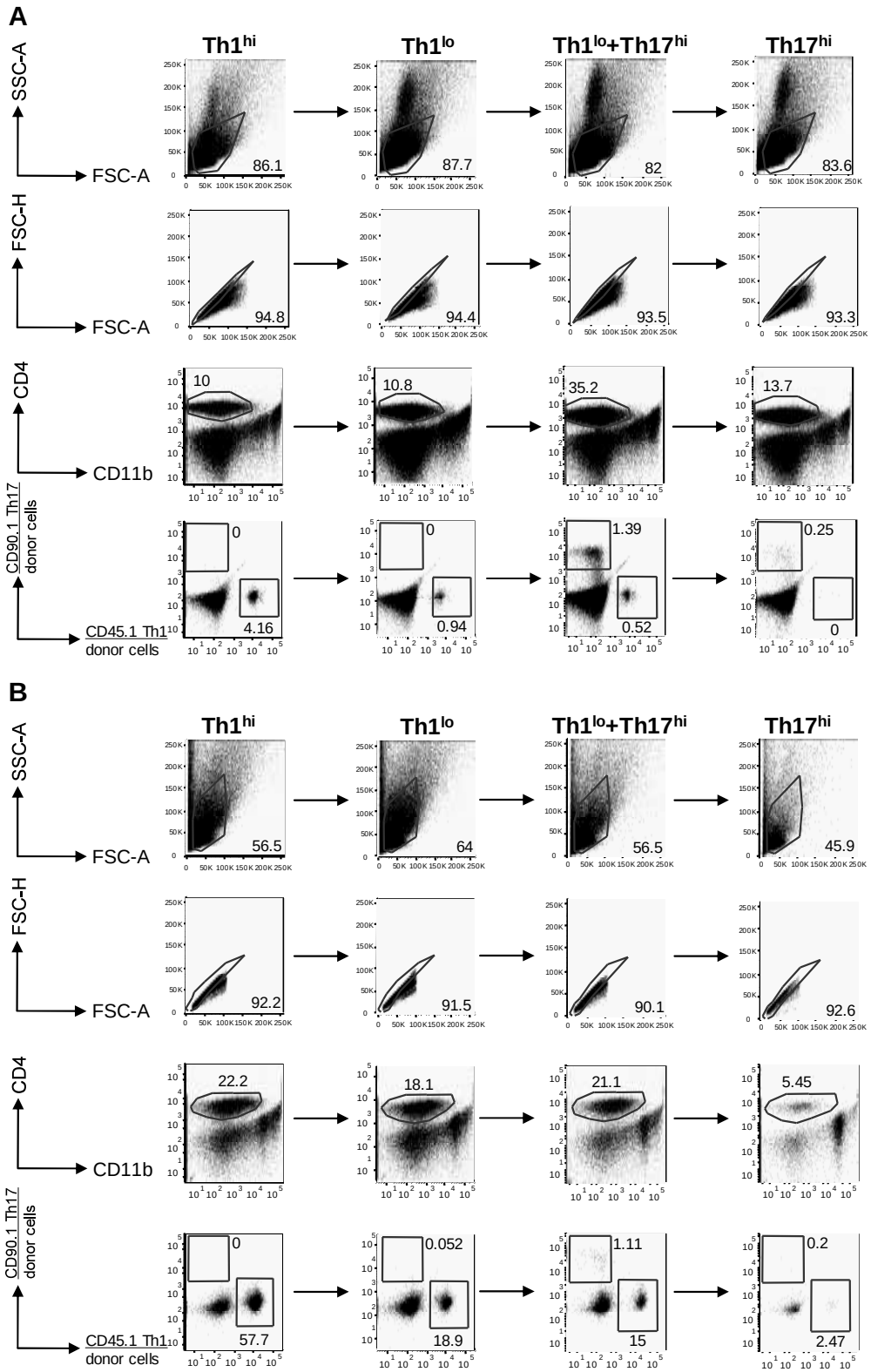


Appendix 5 A Isotype control on Th1 and Th17 polarised cells for Figure 3.11 B.

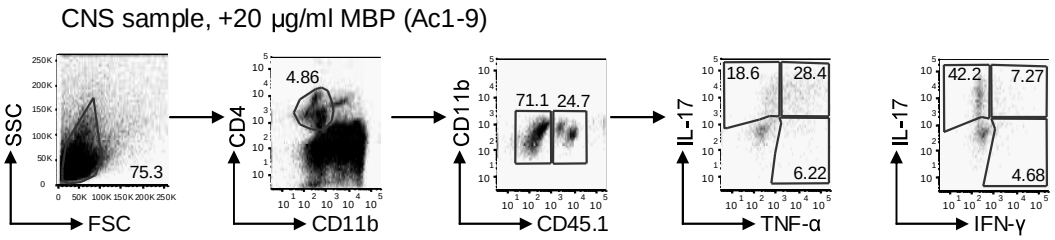
IgG1 FITC was the isotype for IFN-γ, IgG1 APC for IL-17, and IgG1 PE for TNF-α. All flow cytometry cytokine analysis was gated on isotype controls to ensure no non-specific staining. Representative of all isotype control analyses and gating.

Parameters		**Significance**	**p value**
Th1 hi	Th1 lo	*	p=0.0476
Th1 hi	Th1 lo + Th17 hi	ns	p=1.0000
Th1 hi	Th17 hi	**	p=0.0079
Th1 lo	Th1 lo + Th17 hi	ns	p=0.2063
Th1 lo	Th17 hi	ns	p=1.0000
Th1 lo + Th1 hi	Th17 hi	*	p=0.0476

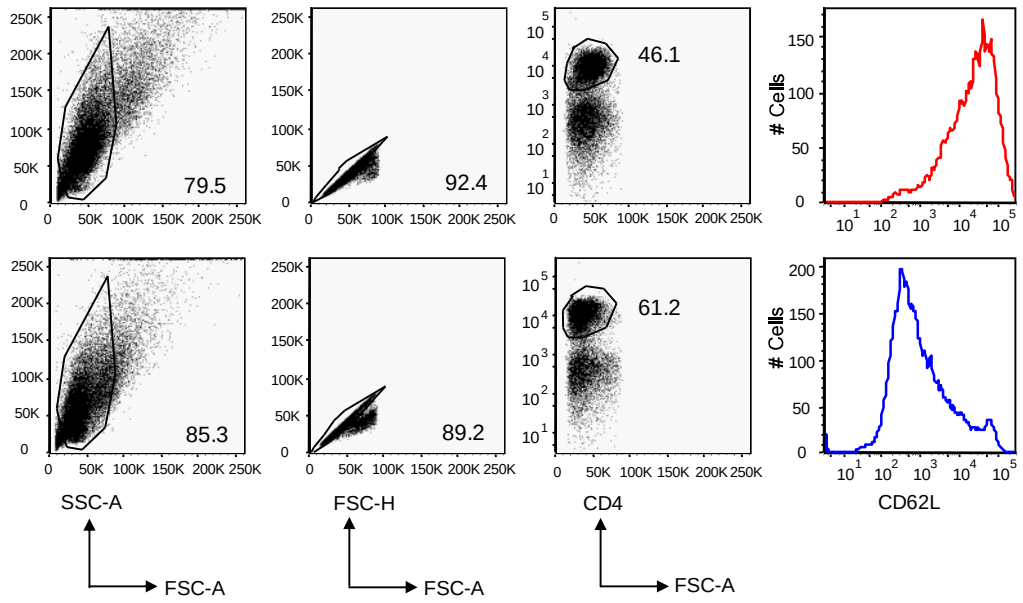
Appendix 5B Fisher's exact test for significant differences in EAE severity for Fig. 3.12 A. As determined by comparing number of mice with EAE maximum score 2 and below, to EAE mice with a score of 3 and above.



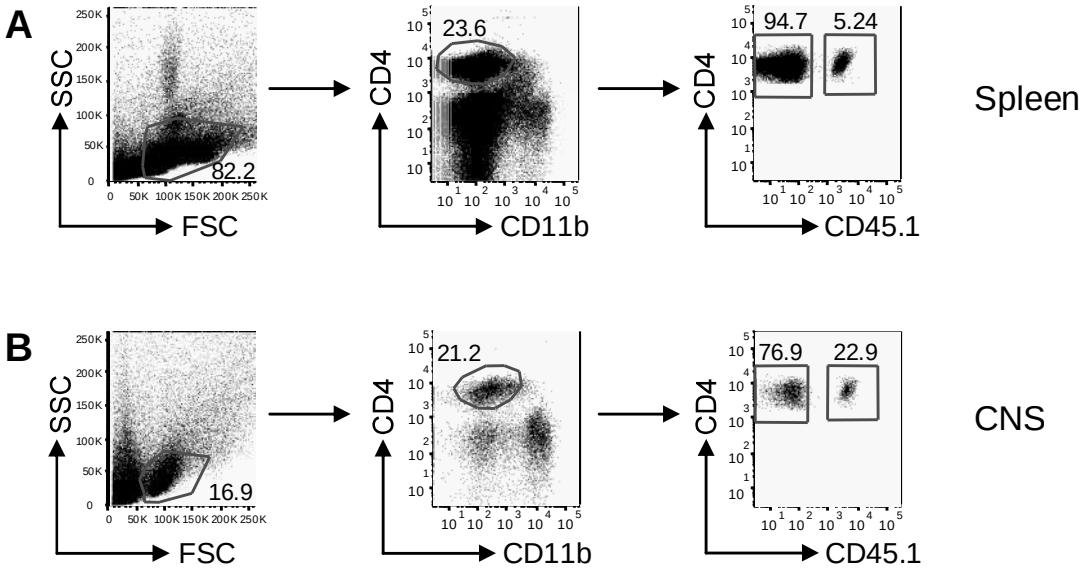
Appendix 5 C. Flow cytometry gating strategy for the location of donor Th1 and Th17 cells for Figure 3.12. Gating strategy to determine location of CD45.1^{+} Th1 donor cells and CD90.1^{+} Th17 donor cells in the **A,** spleen and **B,** CNS in each transfer group of Th1^{hi}, Th1^{lo}, Th1^{lo}+Th17^{hi} and Th17^{hi}.



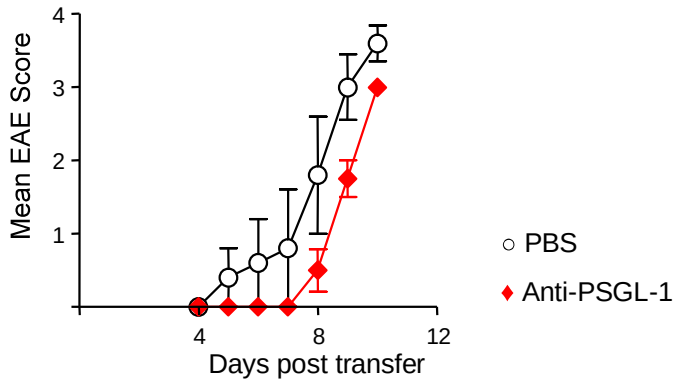
Appendix 6. Flow cytometry gating strategy for location of donor cells and cytokine production for Figure 3.18 A. Gating of CNS sample, highlighting production of IFN-γ, IL-17 and TNF-α in response to MBP (Ac1-9) re-stimulation. Representative of gating for spleen and draining lymph node samples.



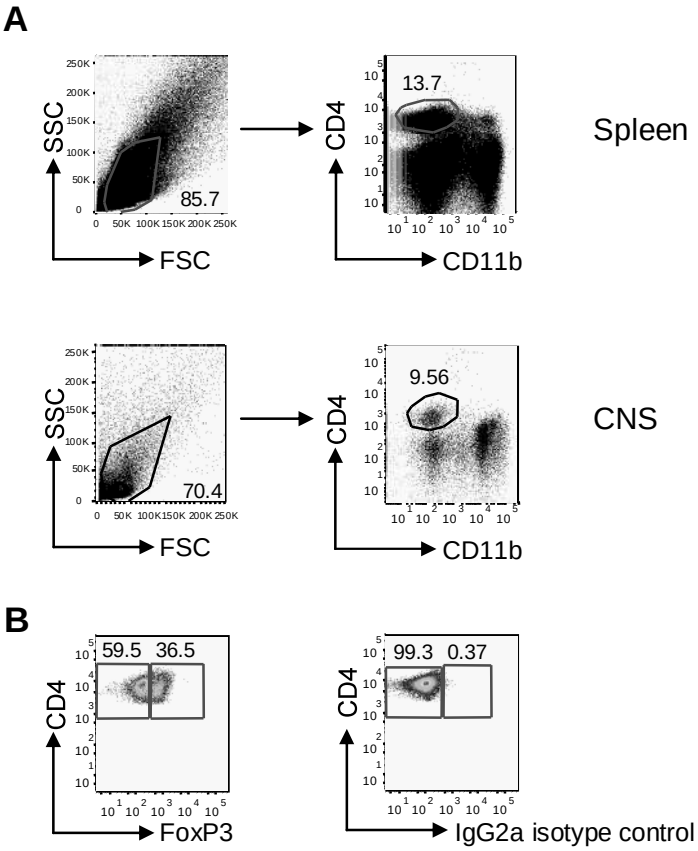
Appendix 7. Flow cytometry gating on Th1 and Th17 cells for phenotypic analysis highlighting CD62L expression for Figure 4.4. Gating strategy for in vitro polarised Th1 cells (top) and Th17 cells (bottom) showing CD62L expression on Th1 (red) versus Th17 (blue) cells. Representative of gating strategy for phenotypic analysis of in vitro polarised cells.



Appendix 8. Flow cytometry gating for location of donor Th1 cells for Figure 4.7. Gating strategy for presence of CD45.1^{+} Th1 donor cells in the **A,** spleen, and **B,** CNS. Representative of gating for all Tg4 passive transfer experiments with the transfer of in vitro polarised Th1 donor cells.



Appendix 9. Observed delay in EAE induction observed after pre-treatment of Tg4 Th1 cells with anti-PSGL-1 as compared to the PBS control. This experiment was sampled at days 4, 7 and 10 post-transfer and therefore the disease course ends at day 10 post-transfer, however the delay in disease induction is still observed, as seen in Figure 4.13.

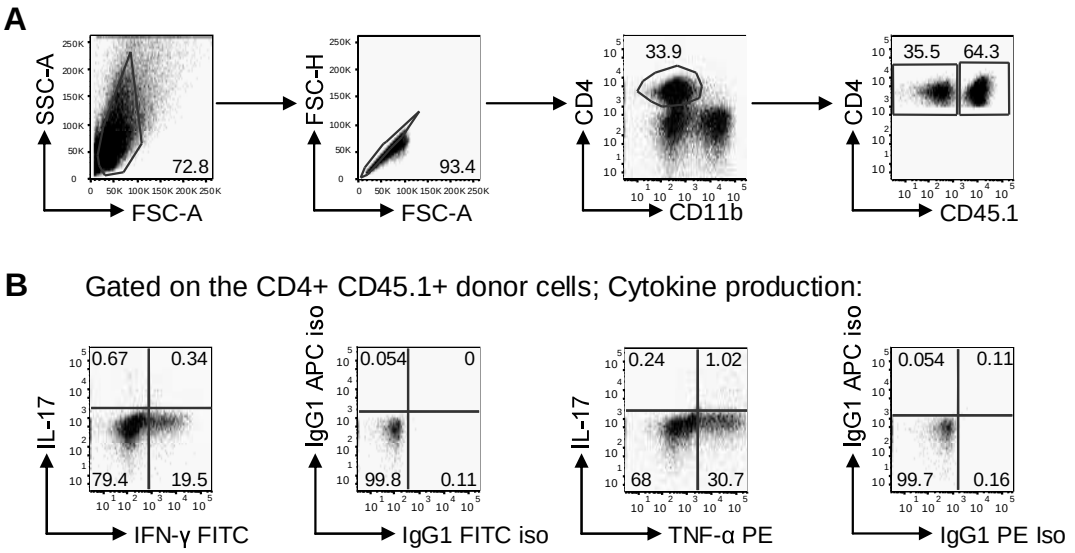


Appendix 10. Flow cytometry gating strategy for determination of CD4$^+$ T cell frequency in spleen and CNS, and FoxP3 expression, for Figure 5.2. A, gating strategy for presence of CD4$^+$ T cells in WT with EAE, in spleen (top) and CNS (bottom), representative of gating for IFN-γ samples; **B,** gating on CD4$^+$ T cells in the WT CNS, FoxP3 expression and the relevant isotype control. Gating always done on isotype controls, and applied to samples. Representative of FoxP3 gating strategy on IFN-γKO CNS samples.

Parameters (A versus B)		Significance	p value
WT Th1	IFN-γKO Th1	ns	p=0.4444
WT Th1	WT Th17	**	p=0.0079
WT Th1	IFN-γKO Th17	**	p=0.0079
IFN-γKO Th1	WT Th17	ns	p=0.1667
IFN-γKO Th1	IFN-γKO Th17	ns	p=0.1667

Appendix 11. Fisher's exact test for significance for EAE scores for Fig. 5.9 A.

As determined for disease severity (number of mice with score 3 and below versus number of mice with score 4 and above for each group).



Appendix 12. Flow cytometry gating for presence of donor Th1 cells in the brain for Figure 5.12. A, gating for presence of CD45.1$^+$ Th1 donor cells in the brain. Gating strategy is representative of that in the spinal cord and spleen for both Tg4 Th1 transfer and Tg4 IFN-γKO Th1 transfer groups. **B,** cytokine production by CD45.1$^+$ donor T cells in terms of IL-17, IFN-γ and TNF-α production showing the relevant isotype controls. Gating always determined on isotype controls and subsequently applied to samples; representative of cytokine gating and cytokine production in the spinal cord and spleen.

Parameters (A versus B)		Significance	p value
WT Th1	IFN-γKO Th1	ns	1.0000
WT Th1	IFN-γKO Th1 IL-17-	ns	1.0000
IFN-γKO Th1	IFN-γKO Th1 IL-17-	ns	1.0000

Appendix 13 A. Fisher's exact test for significance for Classical EAE scores for Fig. 5.15 A. As determined for disease severity (number of mice with score 3 and below versus number of mice with score 4 and above for each group).

Parameters (A versus B)		Significance	p value
WT Th1	IFN-γKO Th1	ns	1.0000
WT Th1	IFN-γKO Th1 IL-17-	ns	0.0769
IFN-γKO Th1	IFN-γKO Th1 IL-17-	ns	0.2821

Appendix 13 B. Fisher's exact test for significance for Atypical EAE scores for Fig. 5.15 A. As determined for disease severity (number of mice with score 3 and below versus number of mice with score 4 and above for each group).

9 Publications

Publications relevant to this thesis are now presented, with permission from the respective publishers.

Immune Cell Entry to Central Nervous System – Current Understanding and Prospective Therapeutic Targets

Catriona T. Prendergast and Stephen M. Anderton*

University of Edinburgh, Centre for Inflammation Research and Centre for Multiple Sclerosis Research, Queen's Medical Research Institute, 47 Little France Crescent, Edinburgh EH16 4TJ. UK

Abstract: Under normal physiological conditions there is minimal entry of immune cells into the central nervous system (CNS) for the purpose of immune surveillance. During inflammation, however, extensive infiltration of immune cells can lead to the induction of CNS autoimmune disease, for example multiple sclerosis (MS) and its animal model experimental autoimmune encephalomyelitis (EAE). The barriers that regulate cellular entry are the blood-brain barrier (BBB) within the CNS parenchyma, and the blood-cerebrospinal fluid (blood-CSF) barrier within the choroid plexus. Understanding how these barriers function to allow the passage of leukocytes from the periphery into the CNS for normal immune surveillance, and under inflammatory conditions, is vital for the development of novel therapeutics targeting immune cell migration in CNS diseases. Contributions from selectins, chemokines, integrins and matrix metalloproteinases allow the migration of leukocytes across the BBB and into the CNS parenchyma. In EAE and MS, the strict maintenance of this process is lost and a large influx of cells is seen. This review focuses on the role of these homing molecules, chemokines and enzymes in the entry of leukocytes into the CNS during inflammatory conditions. It concludes with a model of immune cell entry of the CNS, summarising the current knowledge in this area. Targeting specific molecules to prevent infiltration of inflammatory cells into the CNS could allow disease inhibition without compromising beneficial immune surveillance.

INTRODUCTION

The central nervous system (CNS) was originally considered to be an immune privileged site with the blood-brain barrier (BBB) controlling the entry of lymphocytes into the central nervous system (CNS). However, as hypothesised by Wekerle *et al.* in 1986, under normal physiological conditions, activated lymphocytes are capable of entering the CNS to undertake immune-surveillance [1, 2]. Moreover, under viral or bacterial infection, or inflammatory conditions, large numbers of circulating lymphocytes cross the BBB and gain access to the CNS. Inflammatory cell infiltration of the CNS is clearly an important component of the demyelinating autoimmune disease multiple sclerosis (MS), and its widely used animal model, experimental autoimmune encephalomyelitis (EAE). Although the cause of MS remains unknown, our view of pathogenesis has been moulded by observations in EAE [3]. EAE is mediated by inflammatory $CD4^+$ T cells that infiltrate the CNS, activate resident microglia and recruit inflammatory macrophages; these innate cells are the ultimate effector cells, leading to the destruction of the CNS myelin sheath and oligodendrocyte death. EAE can be actively induced by immunization with complete Freund's adjuvant containing various autoantigens derived from CNS myelin. These include myelin oligodendrocyte glycoprotein (MOG), myelin basic protein (MBP) and proteolipid protein (PLP). Importantly, EAE can also be induced by the transfer of activated encephalitogenic myelin-responsive T cells. This allows studies designed to follow the migration of traceable T cell populations into the CNS to induce EAE.

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BARRIERS TO CNS ENTRY

The CNS derives nutrients from two sources: from the blood vasculature that traverses the meninges and parenchyma, and from the cerebrospinal fluid (CSF). The CSF is made predominantly by the choroid plexus, a richly vascularized invagination of specialized secretory ependymal cells in the lateral, third and fourth cerebral ventricles. The CSF is contained within the sub-arachnoid space (SAS), which is created between the inner pia mater, the arachnoid membrane and the outer dura mater. Of these meningeal membranes, the pia mater and the arachnoid mater together are referred to as the leptomeninges. The SAS provides for circulation of the CSF around the brain and the spinal cord (the CSF turns over three or four times a day). The epithelium of the choroid plexus therefore constitutes the blood-CSF barrier. The blood-brain barrier is formed by the vascular endothelium within the CNS parenchyma [4, 5]. These barriers to entry into the CNS result from the formation of tight junctions between the epithelial cells of the choroid plexus, between the epithelial cells of the arachnoid membrane (together these form the limits of the SAS, i.e., the blood-CSF barrier), and between the vascular endothelial cells found within the CNS parenchyma. These tight junctions severely restrict the egress of macromolecules from the blood into the CSF or CNS interstitial fluid, forcing reliance on trans-cellular transport. Moreover, they provide a very robust obstacle to cellular egress. The capillary endothelial cells of the BBB are surrounded by a basal lamina, pericytes and astrocytic end-feet with microglia also in close attendance. Physiological and pathological changes in the activity of glial cell populations can weaken BBB integrity. Notably,

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inflammatory cytokines such as interleukin (IL)-1β, IL-6 and tumour necrosis factor (TNF)-α can be produced by astrocytes and microglia and can consort to increase BBB permeability.

Therefore the migration of lymphocytes through the brain is usually low, as the endothelial BBB limits the entry into the CNS [2]. In the healthy brain, tight junction components occludin, ZO-1, claudin-3 and claudin-5 are readily detectable. However, during CNS inflammation, for example, EAE, the expression of claudin-3 is lost, specifically in areas of inflammation [6, 7] making the BBB increasingly permeable. This results in an increase in the recruitment of further encephalitogenic cells into the CNS and exacerbation of disease. Of course this inflammation dependent process does not explain how the "pioneer" T cells are able to cross the BBB to initiate the inflammation. In order to develop therapeutic targets to prevent the entry of T cells into the CNS in the inflammation setting, it is important to understand the mechanisms that allow this entry to occur.

When a naïve T cell is activated, it down-regulates molecules required for lymph node entry, for example, CD62L [8] and CCR7 [9]. It also up-regulates those molecules involved in lymphocyte migration to non-lymphoid tissues, for example P-selectin glycoprotein ligand 1 (PSGL-1), tissue-homing integrins and inflammatory chemokine receptors such as CXCR5 [9, 10]. On activation, $CD4^+$ T cells also differentiate into particular effector sub-types, namely T helper (Th) 1, Th2, Th17 or T regulatory cells (Treg). These T helper cell subsets appear to have differing abilities to cross the BBB. This is due to each subset having distinct expression patterns of adhesion molecules and chemokine receptors, resulting in differential homing patterns to sites of inflammation. Th1 cells have been shown to be able to home to, and migrate across, the BBB into the CNS [11]. They are readily found in the CNS of mice with EAE as well as in the CSF of MS patients and in the sites of inflammation, the lesions of MS brains [12]. Recently we have shown that Th17 cells are unable to infiltrate the CNS and initiate EAE whereas Th1 cells are [11]. The entry of Th17 cells into the CNS is currently a matter of debate with some evidence showing they are the first T cells able to enter the CNS during EAE [13], and others showing that they are unable to enter the CNS in the absence of Th1 cells [11]. The entry of Treg into the CNS is also of interest. Treg have been shown to be capable of modulating disease in the brain [14-16] and are therefore a potential route for cellular therapy of CNS autoimmune disease.

SITE OF ENTRY INTO THE CNS

The site of entry into the CNS plays an important role in the ability of lymphocytes to mediate an inflammatory response. The two major routes of entry into the CNS from the blood are *via* the choroid plexus into the CSF, and across the BBB or blood-spinal cord barrier into the parenchymal perivascular space [17]. Entry into the CSF may be important for immunosurveillance of the CNS under normal conditions, whereas entry to the parenchymal spaces of the brain is required during localized inflammatory responses. Migration *via* the choroid plexus involves extravasation from the fenestrated capillary bed into the perivascular spaces, but this must be followed by crossing the tight junctions of the ependymal epithelial layer. In healthy mice, inter-cellular cell adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1) and mucosal addressin cell adhesion molecule-1 (MAdCAM-1) have been found to be important for this process and are localized to the apical surface of choroid plexus epithelial cells, not on the fenestrated capillaries of the choroid plexus [18, 19], and during EAE this expression is increased. Various studies have used intravital microscopy to observe the interaction of encephalitogenic T cell blasts with the endothelial surface [20-22].

MOLECULAR MECHANISMS OF T CELL ENTRY TO THE CNS

The molecular mechanisms defining the entry of lymphocytes into the CNS under normal conditions or during an inflammatory response are still poorly understood. It is very likely that the mechanisms mediating the entry of the first lymphocytes into the CNS are different from those mediating entry once inflammation is established. Cell adhesion molecules (CAMs) are responsible for the recruitment of cells to sites of inflammation. These adhesion molecules can be divided into selectins (e.g. P-selectin, E-selectin); integrins (e.g. leukocyte functional antigens (LFA-1)) and the ligands that bind to the integrins (e.g. ICAM-1, -2, VCAM-1, and platelet/endothelial cell adhesion molecule-1 (PECAM)). Selectins are thought to mediate the initial contact with the endothelium. This interaction is then enhanced by the activation of integrins.

There are thought to be four main steps of leukocyte trans-endothelial migration (Fig. **1**). Firstly, there is an initial low-affinity contact which results in tethering of the lymphocytes and facilitates their rolling along the endothelial cell surface. This is brought about by a transient contact of the endothelium with the lymphocytes, mediated by selectins on the endothelial surface binding to glycosylated ligands on the lymphocyte surface [23]. Secondly, whilst rolling on the endothelium the lymphocytes encounter chemokines. Activation of the corresponding chemokine receptors on the lymphocytes leads to the induction of G protein-linked intracellular signals resulting in the activation of the integrins, altering their conformation from the low to the higher affinity state. In this state, integrins (e.g. LFA-1, and very late antigen (VLA)-4) interact with their appropriate ligands (e.g. ICAM-1 and VCAM-1 respectively) on the surface of the endothelium, providing firm adhesion or arrest of the leukocyte on the endothelium surface. Finally, the leukocyte extravasates across the endothelial layer into the perivascular space. If cells are to enter the parenchyma of the brain or spinal cord, this requires negotiating the tight junctions of the BBB, or the blood-spinal cord barrier, respectively [24] which occurs in an ICAM-1 and ICAM-2 dependent process [25]. In the past it has been suggested that within certain conditions and at certain locations, lymphocytes are capable of migrating across the endothelium by a transcellular route [26-28].

In general, the initial low-affinity contact is thought to be mediated by PSGL-1 on the lymphocyte binding to P and E selectin on the endothelial surface. The higher-affinity contact required for cell arrest and cell diapedesis is thought to

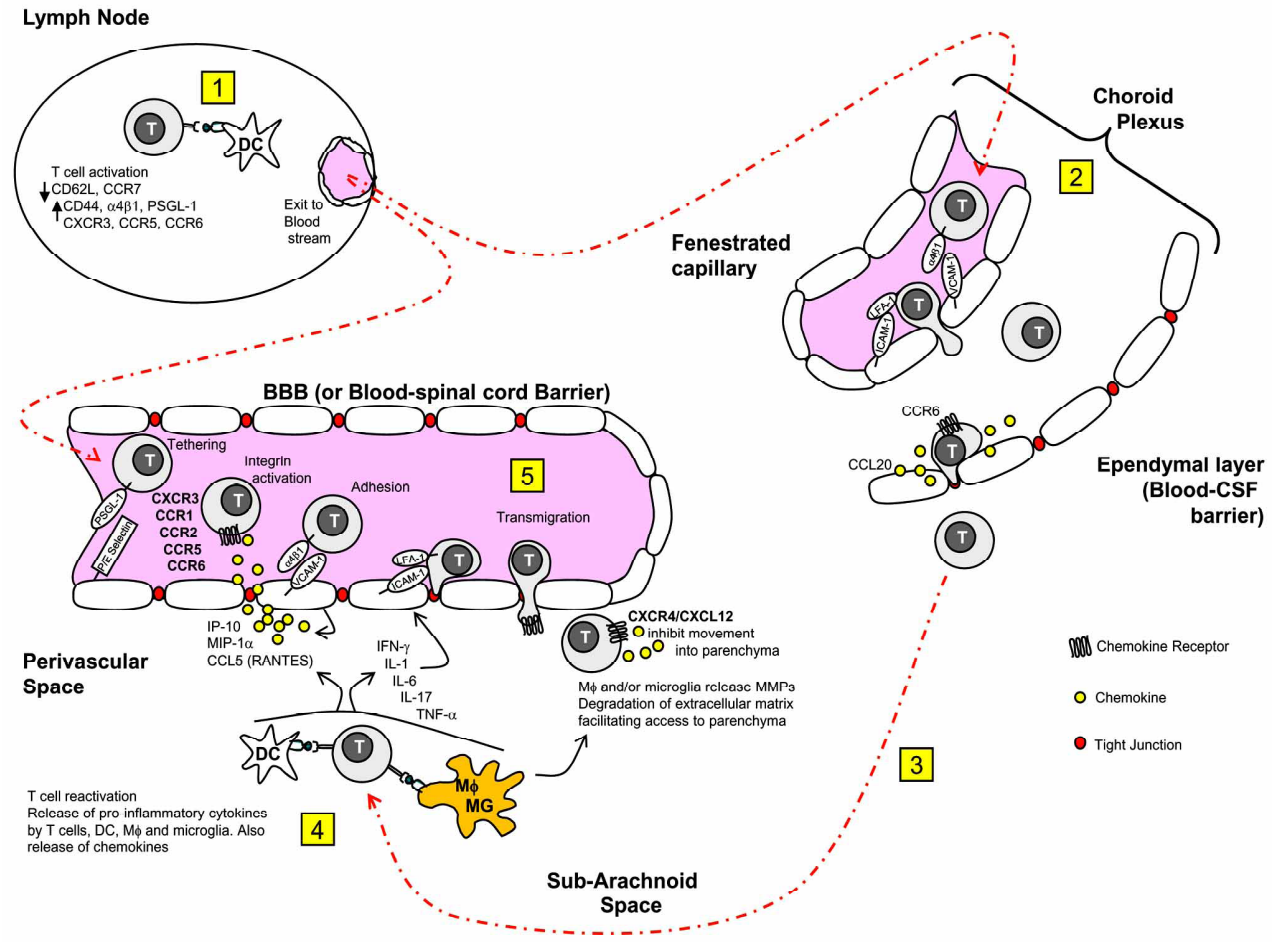

Fig. (1). Multi-step model for autoreactive T cell colonization of the CNS.

1. Myelin responsive T cells are activated in the lymph node by antigen-presenting DC (the antigen may be the myelin autoantigen itself, or a pathogen-derived antigenic mimic). T cells down-regulate lymph node homing molecules (eg CD62L and CCR7) and express integrins and chemokine receptors that will allow access to inflamed tissues. T cells exit the lymph node, into the circulation.

2. First T cells reach the choroid plexus and cross the fenestrated capillaries. Constitutive expression of CCL20 allows $CCR6^{+}$ cells to cross the ependymal layer into the CSF (the precise molecular mechanisms for this, beyond CCR6 expression, remain to be clarified).

3. T cells migrate *via* the CSF through the sub-arachnoid space and possibly into the brain or spinal cord parenchyma.

4. Upon recognition of their cognate myelin antigen - presented by DC, resident microglia (MG), or possibly infiltrating macrophages - T cells are re-activated to produce inflammatory cytokines. These, in addition cytokines produced by DC, MG and macrophages lead to the activation of the local endothelium and expression of adhesion molecules. Chemokines are released by immune cells and stromal/endotheial cells.

5. Circulating T cells bearing the appropriate homing molecules (PSGL-1, α4β1, LFA-1 and a range of chemokine receptors) localize on the CNS endothelium and undergo extravasation into the perivascular space. Innate immune cells (notably inflammatory macrophages will also extravasate). The level of infiltration into the CNS parenchyma is determined by several factors. Its promotion by MMPs produced by activated macrophages or microglia can be balanced by the anti-migratory effects of CXCL12 on $CXCR4^{+}$cells.

be mediated by α4β1-integrin (VLA-4) binding to VCAM-1, or by LFA-1 binding to ICAM -1, -2, or -3.

SELECTINS

The binding of the selectins to their ligands on the lymphocyte surface facilitates lymphocyte rolling across endothelial cell surfaces. Selectins are membrane glycoproteins with distal lectin-like domains, constituting a three-member family of Ca^{2+}-dependent lectins, namely L-, P- and E-selectin. These bind to, and dissociate from, their ligands in a Ca^{2+}-dependent manner [10]. Selectins bind to two types of ligands, mucin-like ligands, for example PSGL-1, and a family of oligasaccharides, known as the lactosaminoglycans. These ligands contain a carbohydrate moiety sulphated sialyl-$Lewis^{x}$.

L-selectin (CD62L) is expressed on most circulating lymphocytes and is important for entry into peripheral lymph nodes. It binds to glycosylation-dependent cell adhesion

molecule 1 (GlyCAM-1) which is constitutively expressed on endothelial cells of the peripheral lymph nodes, as well as to MadCAM-1 on the endothelium of the gut-associated lymphoid tissue. Ligands of L-selectin are also inducibly expressed on endothelial cells at inflammatory sites. There are conflicting data as to whether L-selectin has a role in EAE. Previously it was thought that L-selectin was required for the induction of EAE as CD62L deficient mice were protected from disease induction [29, 30]. Recently however, it has been shown that there is no role for L-selectin in EAE in C57BL/6 or SJL mice [31], suggesting an effect of the genetic background.

E-selectin (CD62E) is expressed on cytokine-activated endothelial cells and P-selectin (CD62P) is stored in the membranes of secretory granules in endothelial cells and is rapidly redistributed to the plasma membrane upon cellular activation by thrombin or histamine. P- and E-selectins expressed on the endothelial cells can bind carbohydrate ligands on lymphocyte populations [32]. The levels of P- and E-selectin expression are regulated by their rate of synthesis, storage and degradation and therefore affect the degree of lymphocyte migration.

P-selectin glycoprotein ligand-1 (PSGL-1) is a 240 kDa homodimer that is capable of binding E-, P- and L-selectin if glycosylated in the correct way [33, 34]. Therefore, although PSGL-1 protein is expressed on all T cells, not all T cells are able to bind selectins (for example, naïve T cells cannot). This is due to the PSGL-1 not being correctly glycosylated and therefore not allowing selectin binding. Glycosyltransferases and sialyl transferases catalyse the production of functional PSGL-1 which is then able to bind to all 3 selectins [34]. Two glycosylation enzymes in particular, core 2 β-1.6-N-acetyl glucosaminyltransferase (C2GnT-I) and α-(1,3)-fucosyltransferase-VII (FucT-VII) are critical for the expression of functional PSGL-1 on T lymphocytes and for PSGL-1 binding to P-selectin [35-38]. Mice lacking either enzyme showed impaired binding of PSGL-1 to P-selectin and subsequently reduced rolling *in vivo* [35, 36].

PSGL-1 expression on Th1 cells and the subsequent binding to P-selectin has been shown to be necessary for the migration of Th1 cells into inflamed skin *in vivo* [39, 40]. Despite Th2 cells expressing the same levels of PSGL-1 protein, this PSGL-1 did not support binding to P-selectin showing that PSGL-1 is important for the entry of Th1 cells specifically into inflamed areas of the skin.

The role of the P-selectin/PSGL-1 interaction in entry of lymphocytes into the CNS appears to be controversial. In humans, P-selectin has been implicated in the entry of lymphocytes into the CSF. Both $CD4^+$ and $CD8^+$ T cells isolated from the CSF of healthy individuals were found to express high levels of PSGL-1 and had evidence of P-selectin binding activity, as well as expression of CCR7 and CD62L on the $CD4^+$ T cells [19, 41]. The site of entry into the CNS appeared to be the choroid plexus and SAS, where interactions between P-selectin/PSGL-1 and ICAM-1/LFA-1 occurred [19]. Using an intravital microscopy model, it was shown that PSGL-1 is needed for the recruitment of mouse autoreactive lymphocytes into the inflamed brain microvessels [42]. In that same model, anti-PSGL-1 antibodies blocked the recruitment of $CD8^+$ T cells in brain vessels of MS patients whereas $CD4^+$ T cell recruitment was not impaired in this setting [41].

Other studies concluded that, despite the expression of functional PSGL-1 on encephalitogenic T cells, it was not required for the development or progression of EAE [43, 44]. C57BL/6 mice deficient in PSGL-1, or those treated with a blocking anti-PSGL-1 antibody did not show a significant reduction in clinical scores along the course of disease, suggesting no obligate role for PSGL-1 in the pathogenesis of EAE.

However, other studies provide data that do imply a role for the P-selectin/PSGL-1 interaction in T cell infiltration of the CNS. IL-12p70 increases T cell expression of the C2GnT-I enzyme [37], thereby increasing functional PSGL-1 binding to P-selectin. That study also showed that incubation of the IL-12-stimulated myelin-reactive $CD4^+$ T cells with a PSGL-1 blocking antibody prior to adoptive transfer resulted in a reduced onset, incidence and severity of EAE [37]. Our own unpublished observations support this view.

IL-12 has also been found to upregulate the expression of CCR5 on T cells in an interferon-γ (IFN-γ) -independent manner [45]. This observed IL-12-dependent expression of PSGL-1 and CCR5, may also have an effect on the migration of encephalitogenic T cells across the BBB, specifically Th1 cells. It has been suggested that blocking reagents against CCR5 or PSGL-1, or agents that antagonise IL-12 may be potential drugs. However, blocking PSGL-1 appears to give conflicting results in different models [37, 43, 44]; the absence of CCR5, as discussed later, does not affect disease induction [46]; and lastly, in the absence of IL-12 (using IL-$12p35^{-/-}$ mice), susceptibility to EAE is not impaired [47]. These data indicate that blocking IL-12 would not be an effective method of preventing the entry of T lymphocytes into the CNS.

INTEGRINS

Integrins are heterodimeric proteins containing α and β chains which, in their high affinity states, mediate firm adhesion of leukocytes to the surface of the endothelium. An example of this is the interaction between ICAM-1 on the endothelial surface binding to lymphocytic LFA-1 ($\alpha_L\beta_2$ integrin) [48]. ICAM-1 has also been found to mediate the binding of neutrophils and macrophages to the endothelium by binding Mac-1 [49]. Endothelial VCAM-1 expression mediates the binding of lymphocytes by interacting with $\alpha_4\beta_1$ integrin (VLA-4), or with α4β7 integrin [50, 51]. $\alpha_4\beta_1$ integrin can also interact with components of the extracellular matrix (for example, fibronectin).

ICAM-1 is expressed on the endothelium surrounding the CNS lesions both in patients with multiple sclerosis and in rodents with EAE [26, 52, 53]. The role of LFA-1 and its ligands ICAM-1 and ICAM-2, in the migration of encephalitogenic T cells across the BBB in EAE is, however, controversial. Using immunocytochemical analysis of frozen sections of brain and spinal cord, the expression of both ICAM-1 and VCAM-1 has found to be upregulated in SJL/J mice during EAE [54]. Numerous studies have implied a role for the LFA-1/ICAM-1 interaction in adhesion and migration

across the BBB [25, 54-58]. LFA-1 is expressed on encephalitogenic T cells and, although this was found not to have a role in the capture and adhesion in the spinal cord microvessels, it did appear to be important for the subsequent transendothelial migration of T cells [25, 57]. This is consistent with the observation that, *in vitro*, cells lacking ICAM-1 and ICAM-2 are still able to support adhesion of T cells to the activated endothelium, but are unable to support transmigration of the T cells across it [58]. These data suggest that the LFA-1/ICAM-1 interaction is more important for the locomotion of the adhered cell across the endothelium, in search of the nearest tight-junction.

The α4β1 integrin/VCAM-1 interaction, on the other hand, is thought to be required for the firm adherence of lymphocytes to the endothelial surface and not for transendothelial migration [59]. Crossing the BBB into the CNS specifically was found to require binding to VCAM-1 as opposed to MAdCAM-1 [60]. It was also reported that, although VCAM-1 binds to both α4β1 and α4β7, only α4β1 integrin was required for the migration of T cells across the BBB during EAE [61]. Using an intravital fluorescence videomicroscopy method, Vajkoczy *et al.* showed the first *in vivo* evidence that an α4β1/VCAM-1 interaction does mediate the capture and subsequent G protein-dependent firm adhesion of T cell blasts to the endothelium [20]. These observations led to the development of α4β1 integrin blockade as a therapeutic option for MS which will be discussed later.

MAdCAM-1 has been shown to be upregulated on the apical surface of the choroid plexus epithelial cells during EAE (as well as ICAM-1 and VCAM-1) [62, 63]. Antibody blockade of MAdCAM-1 binding to its ligand α4β7, prevented EAE development [64]. Faster remission from disease is achieved with combination treatments with anti-MAdCAM-1, VCAM-1 and ICAM-1, however, this same effect is not observed with advanced EAE [64].

Another adhesion molecule found to be required for entry of leukocytes into the CNS is activated leukocyte cell adhesion molecule (ALCAM; CD166) [65]. In both EAE and MS lesions, ALCAM expression is up-regulated on the endothelium of the BBB. In addition, blockade of ALCAM leads to reduced severity and delayed onset of EAE due to an inability of $CD4^+$ lymphocytes and monocytes to enter the CNS by crossing the BBB [66]. In summary, VCAM-1, ICAM-1, MAdCAM-1 and ALCAM are all required for the migration of T lymphocytes into the CNS, either *via* the choroid plexus or *via* the blood-brain barrier. They are, therefore, attractive targets for the prevention of T cells entering the CNS to induce inflammation.

CHEMOKINES AND CHEMOKINE RECEPTORS IN CELL MIGRATION IN TO THE CNS

Chemokines play vital roles in the migration of lymphocytes *in vivo*. Chemokines are small (8-10 kDa) proteins. Their tertiary structure is provided by intramolecular disulphide bonds, and the 4 main families of chemokines are categorised based on the relative location of their cysteine residues. The largest family is the CC chemokines in which the first two of four cysteine residues are adjacent to each other. The other three families of chemokines include the CXC chemokines, in which a single amino acid rests between the cysteine residues; the CX_3C family in which three amino acid residues reside between the cysteine residues, and finally the XC family which only has one cysteine residue. The largest family, the CC chemokines are thought to have a role in attracting mononuclear cells to sites of chronic inflammation. CX_3CL-1 (also called fractalkine) is the only member of the CX_3C family [67]. XCL1 (lymphotactin-alpha) and XCL2 (lymphotactin-beta) are the only members of the XC family of chemokines [68, 69].

Chemokines function by binding their corresponding seven-transmembrane-domain G-protein-coupled receptors [70]. This binding elicits a signalling cascade that results in, for example, activation of the integrins on the cell surface. The response of a cell to chemokine binding is dependent on its pattern of chemokine receptor expression. There are various mechanisms by which chemokines and their receptors can influence the migration of leukocytes across the BBB. The first method is *via* chemokines immobilised on the surface of the endothelium, binding to their respective receptor on the surface of the lymphocyte, resulting in activation of the surface integrins. This leads to the firm adhesion of the leukocyte on the surface of the endothelium. CCL19 (macrophage inflammatory protein (MIP)-3β), CCL20 (MIP-3α) and CCL2 (monocyte chemotactic protein (MCP)-1) have been shown to induce adhesion to the endothelium *via* activated LFA-1 binding to ICAM-1 [71, 72]. CCL-19 and CCL-21 are expressed at the BBB in EAE mice, but not in healthy mice [73]. This would allow entry of $CCR7^+$ T cells [73]. Activated/effector T cells are $CCR7^{lo}$. However, both naïve T cells and central memory T cells are $CCR7^{hi}$. Ligand expression at the BBB might therefore provide a means to attract these cells to the inflamed CNS.

The second mechanism by which chemokines are vital for leukocyte migration across the BBB is by mediating the locomotion of the leukocytes to their nearest endothelial tight-junction. As the leukocyte migrates through the tight junction it is thought to extend chemokine receptor-enriched processes which seek chemokines in the abluminal space [74]. This facilitates their movement through the tight-junction and into the perivascular space. These different roles for chemokines and chemokine receptors make dissecting their specificity and function at each stage challenging, but they also provide encouragement for new therapeutics targeting chemokines.

There is plenty of evidence for the importance of chemokine/chemokine receptor interactions in lymphocyte migration across the BBB (see Table **1**). Because MS and EAE have classically been viewed as Th1-mediated diseases, numerous Th1-associated chemokines and chemokine receptors (for example, CCR5 and CXCR3) were investigated for their expression and possible role in these diseases. In humans, the chemokines interferon-inducible-protein (IP)-10 (CXCL10), MIP-1α (CCL3) and RANTES (regulated upon activation, normal T cell expressed and secreted; CCL5) were found to be up-regulated in the CSF of MS patients during attacks and were also found in MS lesions [12, 75, 76]. Subsequently, looking at specific chemokine receptors it was found that both $CXCR3^+$ and $CCR5^+$ cells accumulated in the lesions [76]. CXCR3 (the IP-10 receptor) was ex-

Table 1. Roles of Chemokines, and their Respective Receptors, in the Migration of Leukocytes into the CNS

Chemokine Receptor	Respective Ligands	Role in Migration	Reference
CCR1	CCL3 (MIP-1α) CCL5 (RANTES)	$CCR1^{-/-}$ mice have milder severity of EAE	[80]
CCR2	CCL2 (MCP-1)	$CCR2^{-/-}$ mice are resistant to EAE induction; CCR2 expression on host cells is required for EAE induction. Blocking CCL2 prevented leukocyte adhesion	[81, 82] [85, 86]
CCR3	CCL5 CCL11	No known role	
CCR4	CCL17 CCL22	No known role	
CCR5	CCL5 (RANTES) CCL3 (MIP-1α)	Upregulated in MS and EAE lesions. $CCR5^{-/-}$ and MIP-$1\alpha^{-/-}$ mice susceptible to EAE Blocking CCL5 prevents leukocyte adhesion	[76] [46] [85, 86]
CCR6	CCL20	In the absence of CCR6, get a delayed onset and/or low severity and incidence of EAE. Increased expression of CCR6/CCL20 specifically at the choroid plexus.	[13, 89, 90] [13]
CCR7	CCL19 CCL21	Expression of CCR7 and its ligands in venules and encephalitogenic T cells in EAE.	[73]
CXCR3	CXCL10 (IP-10)	Upregulated in MS and EAE lesions. Antagonising CXCR3 results in inhibition of EAE.	[73, 76] [77]
CXCR4	CXCL12	Antagonising receptor results in inhibition of EAE. CXCR4/CXCL12 interaction has a role in localising lymphocyte to the perivascular space.	[77] [78]

pressed on lymphocytic cells in the inflammatory infiltrate in active MS lesions. Recently, new evidence for the role of CXCR3 has emerged. Kohler *et al.* showed that antagonising CXCR3 using synthetic receptor antagonists resulted in the inhibition of EAE and the same was found for CXCR4 [77]. However, another novel anti-inflammatory role for CXCR4 and its ligand, CXCL12, has been suggested [78, 79], as discussed later.

CCR1 is needed for optimal leukocyte recruitment in EAE, with $CCR1^{-/-}$ mice displaying a milder severity of disease [80]. In addition, expression of CCR2 on mononuclear cells appears be vital for the induction of EAE, with $CCR2^{-/-}$ mice being resistant to disease [81]. Use of an adoptive transfer model showed that $CCR2^{-/-}$ T cells were pathogenic, but wild-type T cells were unable to induce EAE in $CCR2^{-/-}$ recipients. This highlighted the requirement of CCR2 on the host-derived mononuclear cells for the induction of the disease [81, 82].

CCR5, the receptor of RANTES (CCL5) and MIP-1α (CCL3) was found to be expressed on lymphocytes, macrophages and microglia in active MS lesions [76] suggesting that it may have a role in lymphocyte migration into the CNS. However, C57BL/6 mice deficient in either CCR5 or MIP-1α proved to be susceptible to MOG-induced EAE and showed no differences in the kinetics or severity of disease, or in the infiltration of lymphocytes into the CNS compared to the wild-type mice [83]. It has been shown that CCR5 is required for the entry of Th1 cells into the eye in the model experimental autoimmune uveitis (EAU) [84]. This suggests that CCR5 is important for the infiltration of Th1 cells specifically and that it may be important at the level of transendothelial migration, rather than the initial rolling on the endothelium stage.

The use of blocking antibodies to either CCL2 or CCL5 appears to prevent leukocyte adhesion but not leukocyte rolling, as demonstrated using intravital microscopy [85], as had also been described in a viral model of demyelination [86]. It has recently been shown that the kinin B2 receptor is responsible for modulating the expression of several chemokines, including CCL2 and CCL5, and therefore has a role in the recruitment of leukocytes to sites of inflammation including lesions in the CNS [87].

The expression of CCR6, and its ligand CCL20, has been investigated for their role in lymphocyte entry into the CNS [88]. Both were found to be up-regulated in the spinal cord during EAE. Three recent studies have addressed the function of CCR6 and CCL20 by genetic or pharmacological blockade, with conflicting results. Two studies show essen-

tially total protection from EAE [13, 89] whilst the third reported only delayed onset [90]. Furthermore, the mechanisms underlying these effects conflicted with two reports of impaired migration [13, 90], whereas altered T cell priming in the lymph nodes was implicated in the third study [89]. Perhaps the most interesting of these reports has implicated the CCR6/CCL20 interaction specifically within the choroid plexus as a key check-point in T cell infiltration of the CNS. Small numbers of $CCR6^+$ cells were found to restore susceptibility to EAE in CCR6ko mice. Moreover, intense expression of CCL20 was found in the choroid plexus even in healthy mice and healthy humans, as well as those with MS. Greater numbers of T cells could be found in the choroid plexus of CCR6ko mice than wild type mice that had been primed for EAE [13], implying that an intact CCR6/CCL20 interaction is essential, not for extravasation, but to allow the accumulating pathogenic T cells to cross the tight junctions of the ependymal layer that constitute the blood-CSF barrier.

CCR6 has been shown to be expressed on the newly discovered Th17 subset both in the mouse and in humans [91-93]. The expression of CCR6 on Th17 cells is thought to be vital for their migration into inflamed sites [90, 93, 94]. The importance of CCR6 to the development of EAE would fit with the shift in opinion over the past few years that EAE is a Th17-mediated disease. However, this shift is now under re-appraisal. We have reported that in passive transfer models Th1 cells, but not Th17 cells, can initiate disease [11]. Moreover expression of IL-17 itself is not required for EAE induction [95]. As such, the precise requirements for a T cell to be encephalitogenic in EAE remain to be elucidated. Although CCR6 has been reported to identify Th17 cells, it is not impossible that Th1 cells (or perhaps a subset thereof) can also express CCR6 and that these are responsible for the initial colonization of the CNS through the choroid plexus. Th17 cells and $Foxp3^+$ T regulatory cells have a very close reciprocal relationship regulated by transforming growth factor-β (TGF-β) [96] and both cell populations have been found to express CCR6 [90, 97, 98]. If CCR6 is required for the infiltration of T cells in to the CNS, this suggests that both effector Th17 and regulatory T cells, and perhaps even effector Th1 cells, are able to infiltrate the CNS at the same point. In terms of targeting particular cell types for the prevention of inflammation in the CNS, this would pose a potential specificity problem.

In addition to the classical chemokine receptors, there are also the 'silent' chemokine receptors, for example D6 and Duffy Ag receptor for chemokines (DARC). These chemokine binding molecules have high homology to classical chemokine receptors however they lack the G protein coupling motifs and are therefore unable to elicit a signalling response on binding to a ligand [99]. D6 was thought to have a role in the resolution of inflammatory responses by 'mopping up' inflammation-inducing chemokines. However, surprisingly it was found that $D6^{-/-}$ mice are resistant to the induction of EAE and this is due to impaired generation of T cell responses [100]. Also, it has been suggested that D6 may not be required for the clearance of disease as there is low D6 expression in the spinal cord of healthy mice, and this expression is not up-regulated during EAE.

MIGRATION INTO THE CNS PARENCHYMA

Once the lymphocytes have penetrated the endothelial surface into the perivascular space they still need to infiltrate the brain parenchyma *via* the basement membrane made up of extracellular matrix proteins (laminins, collagen type IV, nidogens and heparin sulphate proteoglycans) [101]. During EAE, leukocytes accumulate within the perivascular space between the endothelial and parenchymal basement membranes leading to the formation of perivascular cuffs. Chemokines and matrix metalloproteinases (MMPs) are required at this stage to allow leukocytes across this area to the CNS parenchyma. In terms of chemokines, CXCL12, the ligand of CXCR4, is found to have a novel anti-inflammatory role [78]. CXCL12 acts to maintain, or localise, CXCR4-expressing mononuclear cells to the perivascular space, thereby preventing their entry into the parenchyma [78]. During EAE however, CXCL12 expression is lost and CXCR4 activation is inhibited. This leads to an increase of migration of infiltrating leukocytes into the CNS parenchyma which subsequently migrate to sites of inflammation and exacerbate inflammation. In this way CXCL12 acts to inhibit the unnecessary infiltration of lymphocytes into the brain parenchyma, and in its absence, for example during inflammation, this level of maintenance is lost. Endothelial basement membranes are also largely made up of laminins and it has been shown that lymphocyte migration in EAE occurs at particular sites in the presence of laminin-α4 and in the absence of laminin-α5 [102]. Recently it has been shown that laminin-α5 selectively inhibits α6β1 integrin (the receptor of laminin-α4) interactions which thereby inhibits lymphocyte migration across laminin-α4 [103]. Laminin-α4-deficient mice showed up-regulation of laminin-α5 and a significant decrease in susceptibility and severity to EAE, showing that infiltrating T cells used this laminin-α4 dependent process to cross the basement membrane. Specifically targeting α6-integrins could therefore be a potential novel therapy to prevent the entry of lymphocytes into the CNS parenchyma.

MMPs are part of a larger class of metalloproteinases (MPs) including the MMPs, a disintegrin and metalloproteinases (ADAMs) and ADAMTS (thrombospondin) groups and these are all inhibited by tissue inhibitor of metalloproteinases (TIMPs). MMPs have a role in numerous processes in normal and pathological conditions and are tightly regulated at various stages including transcription, translation and activation. They are secreted as latent enzymes by macrophages and granulocytes [104]. They therefore require activation before becoming effective proteases. MMPs facilitate the entry of leukocytes into the CNS parenchyma by proteolysis of the extracellular matrix proteins and they have been found to be important mediators of cellular infiltration in CNS inflammation, including in MS and EAE [105-107]. MMPs are capable of attacking the basal lamina which results in more inflammatory leukocytes entering the CNS parenchyma. Consistent with this, inhibition of MMPs in EAE results in lymphocytes being trapped between two basement membranes, after having crossed the endothelial membrane through the tight junctions, but unable to cross into the brain parenchyma [108]. Both MMP-7 and MMP-9 have been

shown to be upregulated in MS patients and in EAE mice [106, 109, 110]. The increased expression of several MMPs in the serum, CSF, CNS and lymphocytes of MS patients would lead to an increase in the number of lymphocytes able to infiltrate the inflamed CNS. Two potential biomarkers for MS have been identified in MMP-2 and MMP-9 [111]. Although several of the 25-member family of MMPs have been shown to be up-regulated in MS and EAE, it has been found that MMP-12 actually has a protective role in EAE [112]. MMP-$12^{-/-}$ mice were found to have the same disease onset as their wild-type counterparts. However, the severity of the relapses was increased [107]. Mice deficient in the MMP-1-inhibitor, TIMP-1, showed more severe myelin pathology associated with EAE, due to increased lymphocyte infiltration and microglial and macrophage accumulation in the brain parenchyma [113]. TIMP-1 therefore has a protective role in limiting the infiltration of inflammatory cells into the parenchyma.

In summary, MMP-mediated degradation of the extracellular matrix is a key process of lymphocyte infiltration into the CNS. The use of MMP inhibitors has highlighted their role in the movement of leukocytes from the perivascular space across the basement membrane of the glia limitans into the CNS parenchyma.

A MODEL

Based on the existing and recent information, it is possible to draw a general model for the recruitment of inflammatory leukocytes into the CNS as follows (Fig. **1**). Encephalitogenic T cells are first activated in the secondary lymphoid organs and, as a consequence, some of these down-regulate lymph node-homing molecules (CD62L and CCR7) whilst upregulating CD44, VLA-4, PSGL-1 and a range of chemokine receptors. These cells therefore exit the lymph nodes into the circulation and as effector T cells first target the choroid plexus, by virtue of its high concentration of CCL20, the ligand for CCR6. Initial arrest of the cells on the choroid plexus endothelium requires the VLA-4/VCAM-1 interaction, with subsequent diapedesis into the extravascular space involving LFA-1/ICAM-1. The autoaggressive T cells then traverse the tight junctions of the ependymal layer (the precise molecular basis for this is unclear), and now have access *via* the CSF and SAS to the brain and spinal cord. Presentation of autoantigen by CNS-resident dendritic cells, or other innate cells such as microglia, perhaps within the leptomeninges, will reactivate the T cells with the subsequent production of inflammatory cytokines such as IL-1β, IL-6, IL-12, TNF-α and IFN-γ (either by the T cells themselves, or by their instruction of the innate immune cells). These inflammatory cytokines have multiple effects on the CNS vascular endothelial cells; affecting the integrity of tight junctions thereby compromising the of BBB or blood-spinal cord barrier and also leading to localised up-regulation of homing molecules such as ICAM-1, VCAM-1, MAdCAM-1 and P-selectin on the luminal surface. These will all allow the further recruitment of effector T cells as well as inflammatory macrophages across the vascular endothelium to establish the classical perivascular lesions seen in EAE and MS. This process can clearly be enhanced by the local production of chemokines; for example IP-10 to attract $CXCR3^+$ Th1 cells, MIP-1α and RANTES to attract $CCR5^+$ cells and CCL2 to attract $CCR2^+$ monocytes/macrophages. The enhanced local production of MMPs will degrade the extracellular matrix, facilitating penetration into the CNS parenchyma that accompanies more severe pathology.

THERAPEUTIC TARGETS

The numerous different molecules that can be targeted to inhibit the unwanted intrusion of inflammatory leukocytes into the CNS, including chemokines and their receptors, integrins, and matrix metalloproteinases, makes targeting migration an attractive and promising approach. A number of therapies have been developed already and several more are in clinical trials at present.

Targeting integrins is a route that has been thoroughly studied and taken advantage of therapeutically. Numerous studies investigated the potential targets including α4β1 [114, 115]. Natalizumab (Tysabri™) is a humanized monoclonal antibody that binds to the alpha-4 integrins α4β1 (VLA-4) and α4β7. This blocks the binding of α4β1 to VCAM-1 at the BBB and blocks the binding of α4β7 to MAdCAM-1. This drug has been successfully used for the treatment of both MS and Crohn's disease. The short term use of the Natalizumab treatment in relapsing-remitting MS (RRMS) resulted in a significant reduction in the number of new active lesions formed as determined by MRI [116, 117]. However, complications of patients developing progressive multifocal leukoencephalopathy (PML), initially when Tysabri™ was given in combination with IFN-β, led to its withdrawal. Tysabri™ has now been re-introduced and follow-up studies have put the risk of PML at ~1/1000. PML is also a complicating issue for other therapies, such as B cell depletion and highlights the need to develop more specific inhibitors of leukocyte migration [118, 119].

Due to the importance of ALCAM/CD6 interactions in co-stimulation in lymphocyte activation and differentiation [120, 121], there may be multiple side effects of using ALCAM as a target to prevent leukocyte entry into the CNS. This therapeutic target does however have advantages over others, as it does not affect the migration of $CD8^+$ T cells into the CNS. This would be needed to prevent CNS viral infections such as JC virus that leads to PML [122].

PSGL-1 is required in the initial tethering and rolling of the leukocytes to the endothelium, and therefore starts the process of adhering the cell to the surface of the endothelium resulting in their eventual migration into the CNS. Inhibition of PSGL-1, though challenging due to its expression on most leukocytes, could present an attractive method of preventing the infiltration of inflammatory leukocytes into the CNS [123].

$CCR5^+$ and $CXCR3^+$ T cells, and their relevant ligands RANTES, MIP-1α and IP-10, accumulate within the CSF in MS patients and EAE mice during disease [75]. Inhibiting these chemokines or their respective receptors could therefore be a potential target for MS therapies. Anti-CCR5 therapy has proved to be effective as an anti-viral treatment in patients with HIV [124, 125]. However, the efficacy of anti-CCR5 treatment to inhibit the infiltration of T cells in to the CNS is still under investigation. There are several anti-CCR2 trials underway for the treatment of MS. Namely, one at

ChemoCentryx, in which phase I has been completed and phase II is planned. Also another CCR2 antagonist is in preclinical trials at Incyte (INCB8696).

CCR6 appears to be an attractive therapeutic target for MS with results showing in the absence of CCR6, mice are highly resistant to the induction of EAE [13, 89]. However, as mentioned earlier, the expression of CCR6 on both Th17 and Treg cells [90] would potentially pose a complication. An anti-CCR6 treatment would prevent the entry of T effector cells (namely Th17 cells, and possibly CCR6-expressing Th1 cells as well) into the CNS which would be a beneficial result, thereby preventing the induction of disease. However, if Treg are also blocked from entering the CNS this effect would be counter-productive. In summary, using CCR6 as a potential therapeutic would be challenging and would require high specificity to distinguish between the cell populations, thus preventing T effector cells from entering the CNS, but still allowing the entry of Treg cells.

Recently kinin receptor B1, a member of the kallikrein-kinin pathway, has been shown to limit EAE pathology. In its absence, kinin receptor B1 deficient mice showed more severe EAE and an increased infiltration of T cells into the CNS [126]. Kinin receptor B1 signalling is thought to specifically regulate the entry of Th17 cells across the BBB endothelium and into the CNS. This suggests that development of kinin receptor B1 agonists could be a potential novel target for MS therapies.

Lastly, several studies have assessed MMPs as potential targets for therapeutic intervention. The use of an MMP-inhibitor, minocycline, inhibited MMP activity. This semi-synthetic tetracycline derivative specifically reduced the production of MMP-9 and the subsequent migration of lymphocytes into the parenchyma, thereby reducing disease severity [127]. This has been extended to studies on its affects in MS patients and recent results using PEG-minocyline liposomes have been promising [128]. In addition, interferon-β, a common immunomodulator in RRMS, has been shown to regulate the levels of MMPs. Interferon-β reduces the levels of MMP-9 production by T cells which subsequently leads to the inhibition of T cell migration into the CNS parenchyma [129, 130]. This could suggest one mechanism of action for interferon-β in RRMS.

CONCLUSION

In conclusion, the blood brain barrier is at the interface between the CNS and the periphery. Under normal physiological conditions, it acts to allow CNS immune surveillance, whilst preventing the entry of unwanted inflammatory cells and pathogens. The migration of leukocytes across the BBB, as shown in this review, is a complex process under strict regulation at each step. During local inflammation, however, the BBB undergoes a structural change that allows a large infiltrate of potentially autoaggressive immune cells into the CNS. Understanding the mechanisms of how and why the BBB undergoes this change is vital. Our current knowledge on BBB structure, leukocyte migration in to the CNS and the nature of those cells may allow the ability to block a specific inflammatory cell subset. There are a wealth of different selectins, chemokines, chemokine receptors, integrins and MMPs that are potential targets for new therapies for CNS autoimmune disease. The challenge is finding a target that is non-redundant, yet specific enough to target particular subsets of lymphocytes that infiltrate the CNS to cause autoimmune inflammation, without compromising essential immune surveillance.

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ABBREVIATIONS

ADAMs	=	A disintegrin and metalloproteinases
ALCAM	=	Activated leukocyte cell adhesion molecule
BBB	=	Blood-brain barrier
C2GnT-I	=	Core 2 β-1.6-N-acetyl glucosaminyltransferase
CAM	=	Cell adhesion molecule
CCL-	=	CC chemokine ligand-
CCR-	=	CC chemokine receptor-
CXCL-	=	CXC chemokine ligand-
CXCR-	=	CXC chemokine receptor-
CNS	=	Central nervous system
CSF	=	Cerebrospinal fluid
DARC	=	Duffy Ag receptor for chemokines
EAE	=	Experimental autoimmune encephalomyelitis
EAU	=	Experimental autoimmune uveitis
FucT-VII	=	α-(1,3)-fucosyltransferase-VII
GlyCAM	=	Glycosylation-dependent cell adhesion molecule
ICAM	=	Inter-cellular cell adhesion molecule
IFN-γ	=	Interferon-γ
IL	=	Interleukin
IP-10	=	Interferon-inducible protein-10
LFA	=	Leukocyte functional antigen
MAdCAM	=	Mucosal addressin cell adhesion molecule
MBP	=	Myelin basic protein
MCP	=	Monocyte chemotactic protein
MIP	=	Macrophage inflammatory protein
MOG	=	Myelin oligodendrocyte protein
MMP	=	Matrix metalloproteinase
MS	=	Multiple sclerosis

PECAM	=	Platelet/endothelial cell adhesion molecule
PLP	=	Proteolipid protein
PML	=	Progressive multifocal leukoencephalopathy
PSGL-1	=	P-selectin glycoprotein-1
RANTES	=	Regulated upon activation, normal T cell expressed and secreted
RRMS	=	Relapsing Remitting Multiple Sclerosis
SAS	=	Sub-arachnoid space
TGF-β	=	Transforming growth factor-β
Th	=	T helper
TIMP	=	Tissue inhibitor of metalloproteinases
TNF-α	=	Tumour necrosis factor-alpha
VCAM	=	Vascular cell adhesion molecule
VLA-4	=	Very late antigen-4

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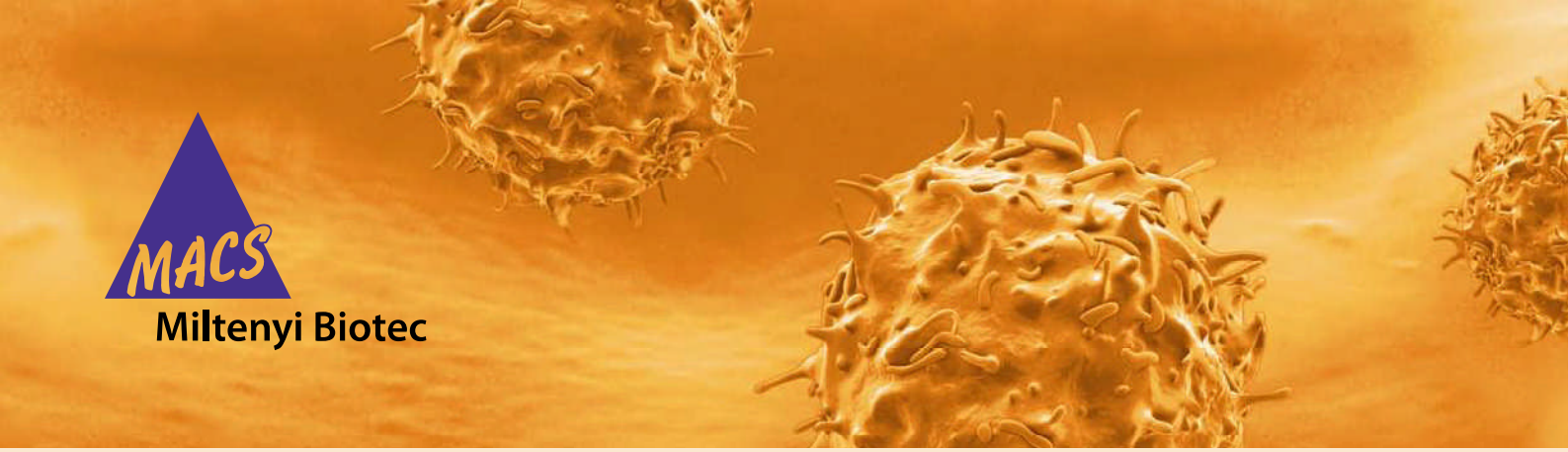

Customer report

Isolation of viable TH17 cells

Detection, isolation, and phenotypic stability of IL-17–producing autoreactive T cells after stimulation with their cognate peptide antigen

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Introduction

The Miltenyi Biotec IL-17 Secretion Assay is optimized for analysis of mouse IL-17–secreting leukocytes after a brief stimulation with PMA and ionomycin, or cognate antigen. This allows viable IL-17–secreting cells to be detected and isolated for further analysis. We are interested in the roles of $CD4^+$ TH1 and TH17 cells in CNS autoimmune inflammation, using experimental autoimmune encephalomyelitis (EAE) as our model[1]. Both TH1 and TH17 cells responsive to myelin autoantigens appear throughout the course of EAE, but the specific roles of these cell populations, in particular the stability of their cytokine production phenotype, are still being elucidated[1-3]. To pursue this question it is vital to generate pure cell populations producing the cytokine of interest, devoid of any contaminating cells. The Mouse IL-17 Secretion Assay by Miltenyi Biotec therefore allows us to study the *in vitro* and *in vivo* stability of IL-17–secreting cells.

Materials and Methods

Mice

In the Tg4 T cell receptor (TCR) transgenic mouse, $CD4^+$ T cells respond to the immunodominant encephalitogenic Ac1-9 peptide of myelin basic protein (MBP) in association with the H-2-A^u MHC class II molecule[4]. B10.PL mice were used as non-transgenic hosts and as a source of antigen-presenting cells (APCs).

Reagents

We made use of the Miltenyi Biotec Mouse IL-17 Secretion Assay – Cell Enrichment and Detection Kit (PE). Data were acquired on a MACSQuant® Analyzer (Miltenyi Biotec) and analyzed using FlowJo™ Software (Tree Star, Inc.; Version 8.5.2).

TH1 and TH17 *in vitro* polarization

We polarized naive Ac1-9–reactive cells (unseparated Tg4 splenocytes) towards the TH1 profile by stimulation with 10 µg/mL Ac1-9 in the presence of 25 ng/mL rIL-12 (R&D Systems) and 25 ng/mL rIL-18 (MBL) polarizing cytokines. To generate TH17 cells naive Ac1-9–reactive cells were stimulated with 10 µg/mL Ac-9 in the presence of 20 ng/mL rIL-6, 20 ng/mL rIL-23 and 3 ng/mL rTGF-β (all R&D Systems). Generation of TH0 populations used peptide stimulation without addition of exogenous cytokines.

Results and discussion

Quantification of IL-17–producing T cells after their restimulation with cognate peptide antigen

As the IL-17 Secretion Assay has been optimized for use with PMA/ionomycin or antigen in unmanipulated T cells, we needed to determine whether we could use this kit in conjunction with our TCR transgenic cells to generate and isolate IL-17–producing cells after stimulation with the cognate Ac1-9 peptide. After polarization for 72 hours we tested the following conditions to restimulate IL-17 production prior to use with the Mouse IL-17 Secretion Assay – Cell Enrichment and Detection Kit:

- medium alone
- 10 ng/mL PMA and 1 µg/mL ionomycin
- 1 µg/mL anti-CD3 antibody
- 10 µg/mL Ac1-9 peptide
- 10 µg/mL Ac1-9 superagonist altered peptide ligand

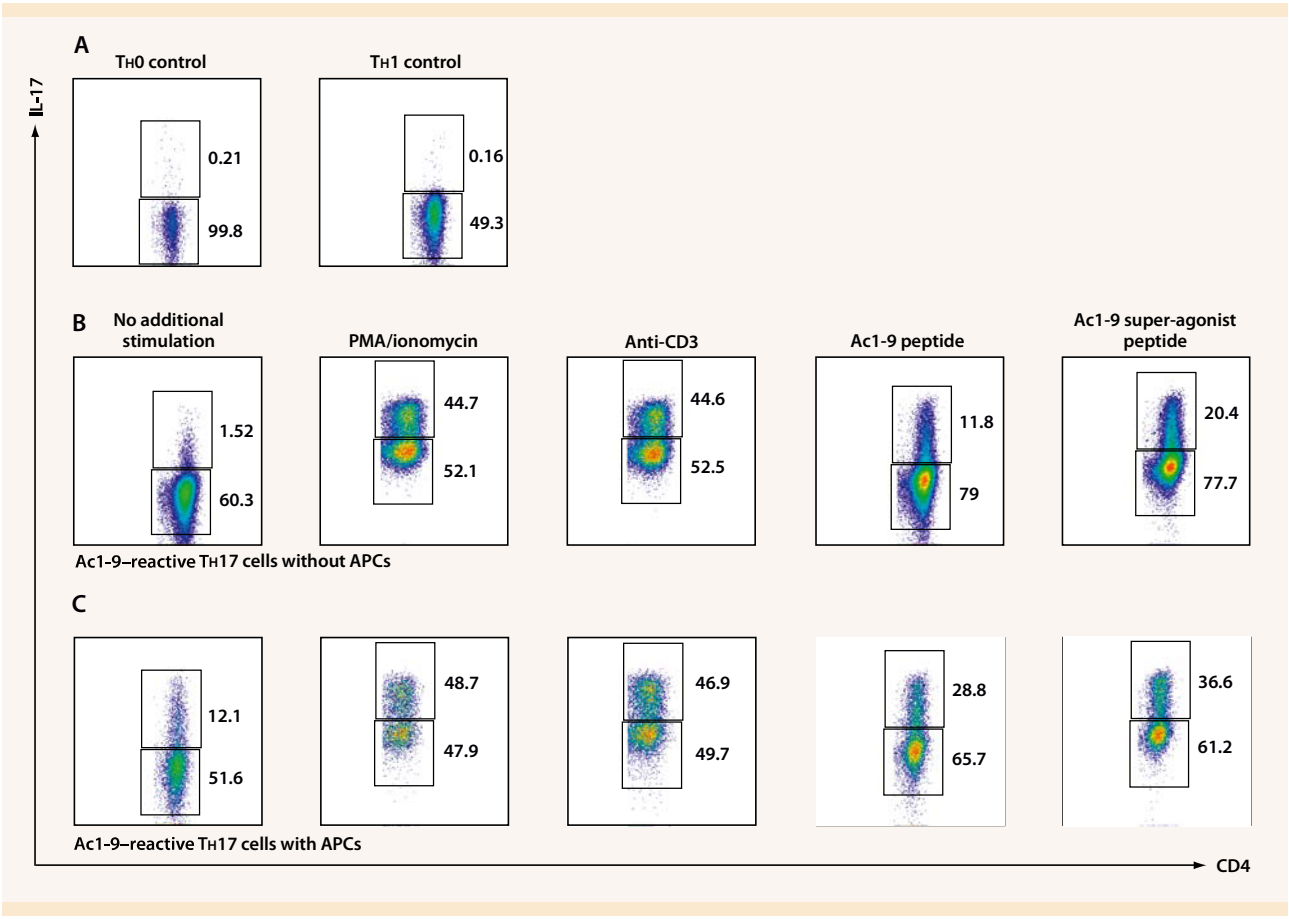

Figure 1. Optimization of the Mouse IL-17 Secretion Assay on Ac1-9–reactive TH17 cells.
Control samples of non-polarized cells (TH0) and TH1–polarized cells with no additional stimulation (A). TH17 cells polarized for 72 hours with Ac1-9 and restimulated with the indicated conditions for 3 hours prior to the IL-17 Secretion Assay without irradiated APCs (B) and with irradiated APCs (C). Gating on the $CD4^+$ cells of each population.

These conditions were tested both in the presence and absence of additional irradiated non-transgenic splenocytes to act as APCs. As control samples TH0 or TH1 cells were used, with no additional restimulation. These produced 0.21% and 0.16% IL-17–producing cells, respectively (fig. 1A, shown as a percentage of the $CD4^+$ population). For the TH17 cells, the basal levels of IL-17 production with no additional stimulation was around 1.5% (fig. 1B). Restimulation either with PMA/ionomycin, or with anti-CD3 for 3 hours at 37 °C led to an up-regulation of $IL\text{-}17^+$ cell frequency (~45%). The whole population of cells appeared to be high for IL-17, but there was a clear separation into $IL\text{-}17^{low}$ and $IL\text{-}17^{high}$ cells.

The above results were obtained without any addition of fresh APCs. Whilst these would not be required for stimulation with PMA/ionomycin or anti-CD3 antibodies, it was conceivable that peptide-driven stimulation would require fresh APCs. However, restimulation with the wild type and superagonist Ac1-9 peptide without fresh APCs gave around 12% and 20% IL-17–producing cells, respectively (fig. 1B). Nevertheless, the addition of APCs increased the peptide-driven $IL\text{-}17^+$ cell frequencies by around 2 to 2.5-fold, without noticeably changing responses to PMA/ionomycin or to anti-CD3 stimulation (fig. 1C). It should be noted, however, that the addition of fresh APCs alone gave a marked increase in "spontaneous" IL-17 production to over 10% of the $CD4^+$ cells. It is currently not clear whether this reflects carryover of antigen from the initial polarization culture, or the production of some soluble IL-17–inducing factor by the APCs. It was important to perform the secretion period of the assay at 37 °C in as large a volume of warm medium as possible to avoid non-specific binding (using no more than 1×10^5 cells/mL).

Sorting of $IL\text{-}17^+$ cells and stability of IL-17 expression

Our next step was to sort the MBP-responsive IL-17–producing cells (fig. 2). TH17-polarized cells were restimulated in the presence of 10 µg/mL Ac1-9, incubated with Anti-IL-17-Biotin and Anti-Biotin-PE. Subsequently, the cells were incubated with the Anti-PE MicroBeads, prior to separation using two MACS® MS Columns. The sorted cells were 99% $IL\text{-}17^+$ (fig. 2C). From a starting population of 1.7×10^7 TH17 cells (of which ~50% were $IL\text{-}17^+$, fig. 2B), 4.56×10^5 $IL\text{-}17^+$ cells were recovered, giving a ~5.5% return. A distinct population of $IL\text{-}17^+$ cells remained in the negative fraction after separation (fig. 2D). Therefore, further optimization should increase the recovery of IL-17–expressing cells.
The $IL\text{-}17^+$ cells were then cultured for 72 hours with plate-bound anti-CD3/anti-CD28, either alone or in combination with varying cytokine conditions to determine their stability

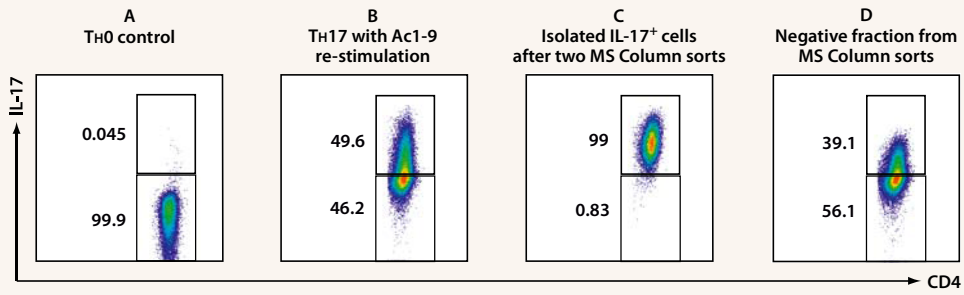

Figure 2. Isolation of IL-17$^+$ TH17 cells using the Mouse IL-17 Secretion Assay – Cell Enrichment and Detection Kit.
TH0 control cells with no additional stimulation (A). Ac1-9–reactive TH17 cells with 10 µg/mL Ac1-9 restimulation (B). Positive fraction of IL-17$^+$ TH17 cells after two MS Column sorts (C). Negative fraction after two MS Column sorts. Gating on CD4$^+$ cells of each population (D).

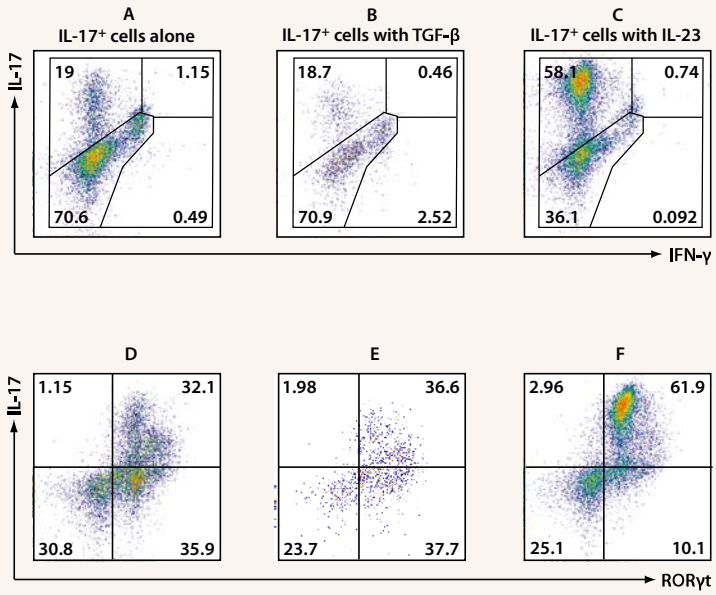

Figure 3. *In vitro* stability of isolated IL-17$^+$ TH17 cells.
IL-17$^+$ cells were cultured in anti-CD3/anti-CD28–coated plates for 72 hours in the presence of medium alone (A), 3 ng/mL TGF-β (B) or 20 ng/mL IL-23 (C) and their stability determined by intracellular staining for IL-17 and IFN-γ. D–F show their corresponding expression of RORγt versus IL-17 under the same conditions as for intracellular staining.

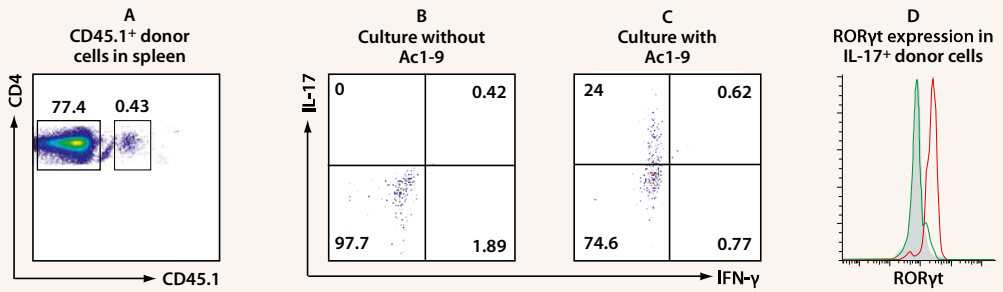

Figure 4. *In vivo* transfer and *ex vivo* analysis of purified IL-17⁺ TH17 cells.
IL-17⁺ cells were sorted from TH17-polarized cultures of CD45.1⁺ cells and transferred into non-transgenic CD45.2 hosts. Lymphoid organs (spleen shown here) were harvested 48 hours later and stained for CD4 and CD45.1 (A). Cells were restimulated overnight without (B) or with (C) 20 µg/mL Ac1-9 peptide prior to intracellular staining for IL-17 and IFN-γ. RORγt expression on CD45.1⁺ donor cells (red line), CD45.2⁺ host cells (green line) and isotype control (filled histogram) (D). B–D are gated on CD4⁺CD45.1⁺ cells to focus on transferred MBP-responsive cells.

in vitro in terms of IL-17 versus IFN-γ production (Fig. 3). Inclusion of TGF-β did not alter the cytokine profile significantly (fig. 3A, B). The addition of IL-23, however, allowed the cells to maintain their IL-17 production profile (58% versus 19% without IL-23) without triggering IFN-γ production (fig. 3A, C). In contrast, in the presence of IL-12 and IL-18, the expression of IL-17 was reduced, with a concomitant appearance of IFN-γ production (data not shown).

These results were reflected by the expression of RORγt (fig. 3D–F), with the addition of IL-23 maintaining the IL-17⁺RORγt⁺ phenotype of the TH17 cells (fig. 3F).

In order to undertake a preliminary test of *in vivo* stability, CD45.1⁺ transgenic TH17 cells were polarized. The IL-17⁺ cells were subsequently sorted and transferred by intravenous injection into CD45.2 non-transgenic recipients. After 48 hours the transferred CD45.1 cells could be identified in the spleen (fig. 4A) and lymph nodes (data not shown). Upon overnight *ex vivo* culture in the absence of peptide stimulation, the donor cells did not produce any IL-17 (fig. 4B). In contrast, inclusion of Ac1-9 reactivated the transferred cells' capacity to produce IL-17, without any IFN-γ production (fig. 4C). The transferred cells also maintained their RORγt expression (fig. 4D).
As shown here, the IL-17 Secretion Assay works well with the use of specific antigens for stimulating IL-17 production by TH17-polarized cells in our model. It should therefore be readily adaptable for other studies requiring antigen-driven T cell activation. It allows the isolation of pure populations of IL-17⁺ cells for downstream *in vitro* and *in vivo* analyses. In relation to the current interest surrounding TH17/TH1 interconversion[1,3], it will be important to incorporate anti-IFN-γ to allow isolation of IL-17⁺IFN-γ⁻ and IFN-γ⁺IL-17⁻ cells. This was not performed here.

In conclusion, the Miltenyi Biotec Mouse IL-17 Secretion Assay – Cell Enrichment and Detection Kit will prove to be a useful tool for the isolation of viable IL-17⁺ cells for further analysis both *in vivo* and *in vitro*.

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Cutting Edge

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Cutting Edge: Th1 Cells Facilitate the Entry of Th17 Cells to the Central Nervous System during Experimental Autoimmune Encephalomyelitis[1]

Richard A. O'Connor,* Catriona T. Prendergast,[2]* Catherine A. Sabatos,[2]† Clement W. Z. Lau,[2]* Melanie D. Leech,* David C. Wraith,† and Stephen M. Anderton[3]*‡

It has recently been proposed that experimental autoimmune encephalomyelitis, once considered the classical Th1 disease, is predominantly Th17 driven. In this study we show that myelin-reactive Th1 preparations devoid of contaminating IL-17$^+$ cells are highly pathogenic. In contrast, Th17 preparations lacking IFN-γ^+ cells do not cause disease. Our key observation is that only Th1 cells can access the noninflamed CNS. Once Th1 cells establish the experimental autoimmune encephalomyelitis lesion, Th17 cells appear in the CNS. These data shed important new light on the ability of Th1 vs Th17 cells to access inflamed vs normal tissue. Because the IL-17-triggered release of chemokines by stromal cells could attract many other immune cells, allowing Th17 cells to access the tissues only under conditions of inflammation may be a key process limiting (auto)immune pathology. This has major implications for the design of therapeutic interventions, many of which are now aiming at Th17 rather than Th1 cells. *The Journal of Immunology,* **2008, 181: 3750–3754.**

Experimental autoimmune encephalomyelitis (EAE)[4] had long been considered the prototypic Th1-mediated autoimmune disease (1, 2) until recent findings suggested a primary role for Th17 cells in this model (3, 4). This paradigm shift has sparked a rapid and remarkable change in emphasis in the search for disease-modifying drugs away from the Th1 pathway toward the Th17 pathway (5–8). It is therefore important to have a clear understanding of the relevance of each of these pathways in autoimmune pathology. This requires an assessment of the ability of Th1 or Th17 cells to initiate pathology upon adoptive transfer in the absence of each other.

With the purity of the Th phenotype in mind, we have evaluated the EAE-initiating capacity of Th1 vs Th17 populations. Our results indicate that Th1 cells have a preferential ability to access the noninflamed CNS and that the pathology they cause promotes the subsequent infiltration of Th17 cells.

Materials and Methods

Mice, Ag, and tissue culture medium

C57BL/6 mice (CD45.2/CD90.2, CD45.1/CD90.2, or CD45.2/CD90.1), Tg4 × CD45.1 mice, and B10.PL mice were bred under specific pathogen-free conditions at the University of Edinburgh (Edinburgh, U.K.). Tg4 and B10.PL × RAG2$^{-/-}$ mice were bred under specific pathogen-free conditions at the University of Bristol (Bristol, U.K.). Experiments received University of Edinburgh/University of Bristol ethical approval and were performed under U.K. legislation. The myelin oligodendrocyte glycoprotein peptide 35–55 (pMOG; MEVGWYRSPFSRVVHLYRNGK) and myelin basic protein (MBP) acetylated (Ac) peptide Ac1–9 (Ac-ASQKRPSQR) were synthesized by the Advanced Biotechnology Centre, Imperial College (London, U.K.). The tissue culture medium was RPMI 1640 medium supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 5 × 10-5 M 2-ME (all from Invitrogen) and 5% FCS (Sigma-Aldrich).

Cell culture and passive EAE

C57BL/6 mice were immunized s.c. with 100 μg of pMOG in CFA. Ten days later, draining lymph node cells were recovered and cultured with 10 μg/ml pMOG for a total of 72 h. For Th1 polarization, 25 ng/ml rIL-12 (R & D Systems) and 25 ng/ml rIL-18 (MBL) were used. rIL-2 (0.5 ng/ml; R and D Systems) was added at the start of Th1-polarizing cultures, and the concentration of IL-2 was raised to 2.5 ng/ml after 48 h. For Th17-polarization, 20 ng/ml rIL-6, 20 ng/ml rIL-23, and 3 ng/ml rTGF-β (all from R & D Systems) were used. After 72 h, 4 × 10^6 blasts were injected i.v.

FACS-sorted CD4$^+$CD62L^{high}CD25$^-$ Tg4 TCR transgenic cells were cultured for 72 h with irradiated B10.PL splenocytes and 10 μg/ml MBP Ac1–9 using the Th1- and Th17-polarizing conditions described above. After 72 h, 3 × 10^6 blasts were injected i.v. For transfer to B10.PL × RAG2$^{-/-}$ recipients, naive Tg4 splenic and lymph node populations were stimulated with 10 μg/ml Ac1–9 under either Th1-polarizing conditions (5 ng/ml IL-12, and 10 μg/ml anti-IL-4), or Th17-polarizing conditions (3 ng/ml TGF-β, 20 ng/ml IL-6, 20

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[4] Abbreviations used in this paper: EAE, experimental autoimmune encephalomyelitis; Ac, acetylated; MBP, myelin basic protein; pMOG, myelin oligodendrocyte glycoprotein peptide.

ng/ml IL-23, and 3 μg/ml anti-IFN-γ) for 5 days. IL-2 (20 U/ml for Th1-polarization and 10 U/ml for Th17-polarization) was included from day 3. After 5 days of culture, 3×10^6 blasts were injected i.v. On the day of transfer, each mouse also received 200 ng of pertussis toxin. Clinical signs of EAE were assessed as described previously (9).

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All Abs were from eBioscience except for anti-CD4-PerCP (BD Pharmingen) and anti-IL-17-allophycocyanin (BioLegend). Intracellular cytokine staining of peptide-stimulated cells was performed as previously described (9). For staining at the end of polarizing cultures, 1 μg/ml PMA, 50 ng/ml ionomycin, and 1 μl/ml brefeldin A was added for the last 4 h of culture.

Analysis of cytokine secretion

Ag-induced cytokine production by lymph node cells (6×10^5 per 200 μl per well) was assessed by ELISA. Polarized T cells (2×10^4 per 200 μl per well) were extensively washed before restimulation with irradiated splenocytes (2×10^5/well) and a dose range of Ag as indicated. After 72 h, cytokine concentration was determined by ELISA or cytometric bead array using an inflammation array (BD Pharmingen) or a Th1/Th2 10plex kit (Bender MedSystems) according to the manufacturer's instructions.

Real-time PCR

RNA was extracted after 72 h of culture in the presence or absence of polarizing cytokines. Primer sequences used for ROR-γt and T-bet have been published previously (10). Expression was normalized to hypoxanthine phosphoribosyltransferase and expressed as the fold induction over cells stimulated under neutral conditions.

Statistical analysis

Statistical analysis of results was performed using the Mann-Whitney *U* test and the two-tailed Student's *t* test.

Results

Th1 cells initiate EAE upon passive transfer and promote the recruitment of Th17 cells to the CNS

Using draining lymph nodes from mice immunized with the pMOG peptide 35–55, our emphasis was to generate the cleanest possible Th1 and Th17 preparations. Stimulation with pMOG alone induced a mixed response dominated by IFN-γ production and a small proportion of cells producing IL-17 or coproducing IFN-γ and IL-17 (Fig. 1*A*). The addition of IL-12 and IL-18 maximized IFN-γ production and repressed IL-17 production, giving the highest ratio of IFN-γ to IL-17 producers (Fig. 1*A*). Although IL-23 induced the highest frequency of IL-17-producers (~40% of cells), it did not reduce the overall proportion of IFN-γ producers (~20% of cells made IFN-γ, either alone or in combination with IL-17). TGF-β was required to suppress IFN-γ production. The IL-6 plus IL-23 plus TGF-β combination therefore produced the most highly polarized Th17 population (Fig. 1*A*). As exemplified in Fig. 1*B*, administration of Th1 cells alone or Th1 together with Th17 cells led to clinical disease, whereas Th17 cells alone were not pathogenic. Similarly, populations generated in the presence of IL-6 and TGF-β (no IL-23) did not cause EAE alone and did not significantly modulate the course of disease when cotransferred with Th1 cells (Fig. 1*C*).

To assess their comparative capacities to home to the target organ, Th1-polarized and Th17-polarized cells were sourced from CD45.1/CD90.2 donors and CD45.2/CD90.1 donors, respectively, for injection into CD45.2 CD90.2 hosts. Both Th1 and Th17 populations were clearly identifiable in the spleen (Fig. 2*A*) with Th17 preparations at significantly higher frequencies than Th1 preparations when transferred either singly or in tandem (Fig. 2*A*). The picture from the CNS (Fig. 2*D*) was the reverse of that from the spleen. A strong Th1 infiltrate

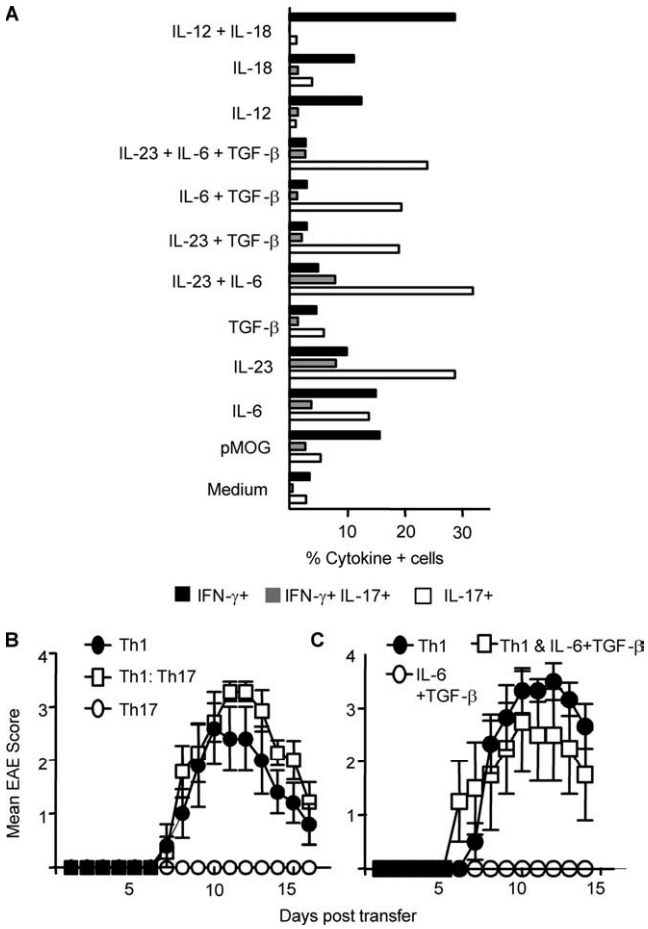

FIGURE 1. Polyclonal Th1 but not Th17 effectors initially activated in vivo induce disease upon passive transfer. Draining lymph node cells from pMOG-immunized mice were restimulated for 72 h with pMOG under Th1- or Th17-polarizing conditions. *A*, The proportion of $CD4^+$ T cells capable of producing IFN-γ (black bars), IL-17 (open bars), or IFN-γ and IL-17 (gray bars) after 3 days of culture in the presence of pMOG and the indicated recombinant cytokines. *B*, EAE score in recipients of Th1- (closed circles), Th17- (open circles), or Th1- and Th17-polarized (open squares) cells. Data (n = 5–7 mice per group) are from one of three experiments giving consistent results. *C*, EAE score in recipients of Th1- (closed circles) and IL-6 plus TGF-β-polarized cells (open circles) or Th1- and IL-6 plus TGF-β-polarized cells (open squares) (n = 3–6 mice per group).

was clearly identifiable (~100-fold enriched as a percentage of total $CD4^+$ cells compared with the spleen). Th17 cells were not identifiable in the CNS if transferred alone but were present when transferred together with Th1 effectors (Fig. 2*D*). We conclude that Th1 lymphocytes have a superior capacity to home to the CNS and orchestrate inflammation and cellular recruitment, thereby facilitating the entry of Th17 cells.

The polarization of pMOG-reactive donor populations is stable

The stability of the cytokine phenotype confirmed that the disease seen with Th1 populations was not because of residual IL-17 production and that the lack of disease with Th17 preparations did not reflect a loss of IL-17 production. Among the cells recovered from the spleen and the CNS, the phenotypes of both populations appeared stable (Fig. 2, *E–H*). It is important to emphasize that donor Th1 cells extracted from the CNS of diseased mice did not show any capacity for IL-17 production (Fig. 2*H*).

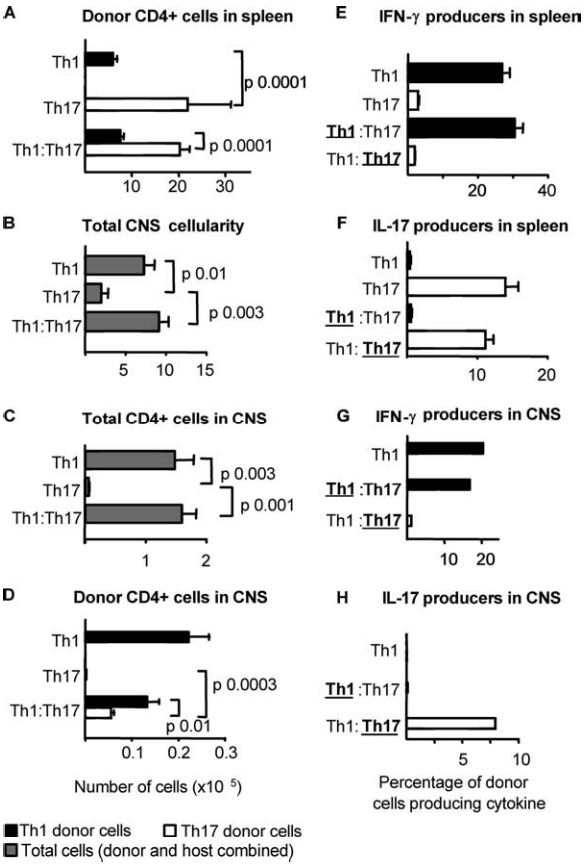

FIGURE 2. Th1- but not Th17-polarized effectors enter the CNS and initiate inflammation. Polyclonal Th1- (CD45.1/CD90.2) and Th17-polarized (CD45.2/CD90.1) cells generated from the draining lymph nodes of pMOG-immunized mice were transferred to CD45.2/CD90.2 hosts alone (Th1, Th17) or in combination at a 1:1 ratio (Th1:Th17). The distribution of donor cells on day 16 after transfer was assessed by FACS analysis. *A*, The number of Th1- (closed bars) and Th17-polarized (open bars) cells recovered from the spleen of recipient mice. *B*, Total number of mononuclear cells recovered from the CNS of recipient mice. *C*, Total number of $CD4^+$ T cells recovered from the CNS (donor and host cells combined). *D*, Number of donor Th1 (closed bars) and Th17 (open bars) recovered from the CNS. *E–H*, Individual splenic populations (*E* and *F*) or pooled CNS mononuclear cells (*G* and *H*) sampled from recipient mice were stimulated overnight with pMOG, and cytokine production by donor cells originally polarized to Th1 cells (closed bars) and Th17 cells (open bars) was assayed by intracellular cytokine staining with IFN-γ (*E* and *G*) and IL-17 (*F* and *H*). Bars represent the mean ± SE of 5–7 recipient mice per group. In the cotransferred groups shown in panels *E–H*, the populations analyzed for cytokine production are identified with boldface type and are underlined.

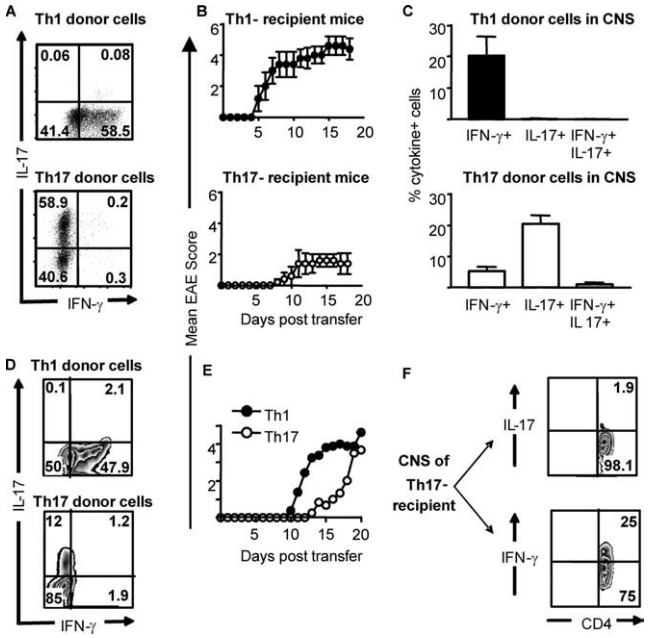

FIGURE 3. Th17-polarized cells from MBP-reactive TCR transgenic mice can induce mild/delayed EAE, correlating with the in vivo appearance of IFN-γ-producing cells. Th1- and Th17-polarized populations were generated from naive Tg4 mice and transferred into B10.PL mice (*A–C*) or B10.PL × $RAG2^{-/-}$ mice (*D–F*). *A* and *D*, Intracellular cytokine staining of Th1-polarized (*top panels*) and Th17-polarized (*lower panels*) Tg4 cells before transfer. *B*, Clinical course of EAE induced by the transfer of Th1 (*upper panel*) and Th17 (*lower panel*) cells, $n = 5$. *C*, Ac1–9 stimulated IFN-γ and IL-17 production by Th1 (*upper panel*) and Th17 (*lower panel*) donor cells recovered from the CNS as determined by intracellular cytokine staining. Graphs show the mean ± SD of cells recovered from three surviving Th1-transferred and five Th17-transferred mice. *E*, Th1 (closed symbols), or Th17-polarized (open symbols) Tg4 T cells (as shown in *D*) were transferred to B10.PL × $RAG^{-/-}$ recipients (three to four mice per group). *F*, Ac1–9-stimulated cytokine production by $CD4^+$ T cells recovered from the CNS of a representative B10.PL × $RAG2^{-/-}$ recipient with a disease score of 3. Data are from two of seven experiments giving consistent results.

Th17-polarized, myelin-reactive TCR transgenic T cells can induce EAE, but only in the presence of IFN-γ-producing cells

The current understanding that IL-6 plus TGF-β is the key combination for driving Th17 differentiation of naive cells has come from either anti-CD3 stimulation of polyclonal populations or Ag stimulation of naive TCR transgenic cells. To date, the use of naive T cells differentiated in the presence of these cytokines has not been described in an EAE model. We made use of the Tg4 mouse that expresses a transgenic TCR recognizing the Ac1–9 peptide of MBP (11). We generated Th1- and Th17-polarized populations by primary in vitro stimulation of $CD4^+CD62L^{high}CD25^-$ cells taken from Tg4 × CD45.1 mice for transfer into B10.PL (CD45.2) recipients (Fig. 3*A*). Th1-polarized cells (devoid of IL-17 producers) efficiently induced severe, often fatal EAE (Fig. 3*B*). The number of Th1 blasts transferred correlated well with the severity of EAE. EAE could be induced with as few as 1×10^6 Th1 blasts. Numbers beyond 4×10^6 led to high mortality (data not shown). In this model, Th17-polarized populations could occasionally induce some EAE, but this was a mild disease with delayed onset and reduced severity (Fig. 3*B*). Furthermore, despite stringent Th17 polarization pretransfer (Fig. 3*A*), those donor cells recovered from the CNS consistently displayed a capacity for IFN-γ production (Fig. 3*C*). There was a striking shift in the $IL\text{-}17^+$:$IFN\text{-}\gamma^+$ ratio in these populations from ~200:1 at the time of transfer to ~3:1 upon extraction from the CNS. In contrast, Th1 donor cells recovered from the CNS did not show any capacity for IL-17 production upon restimulation ex vivo (Fig. 3*C*). The Th1 phenotype was very stable during preclinical stages, through to the peak of disease (days 5–14; data not shown). In contrast. "Th17" cells recovered from the CNS had a propensity for IFN-γ production, suggesting a degree of instability upon reencounter with Ag (discussed below).

The data shown in Fig. 2 indicated that transferred IL-17 producing cells did not have an inherent fragility in vivo (they accumulated in large numbers in the spleen). Nevertheless, we sought to maximize their opportunity for clonal expansion and

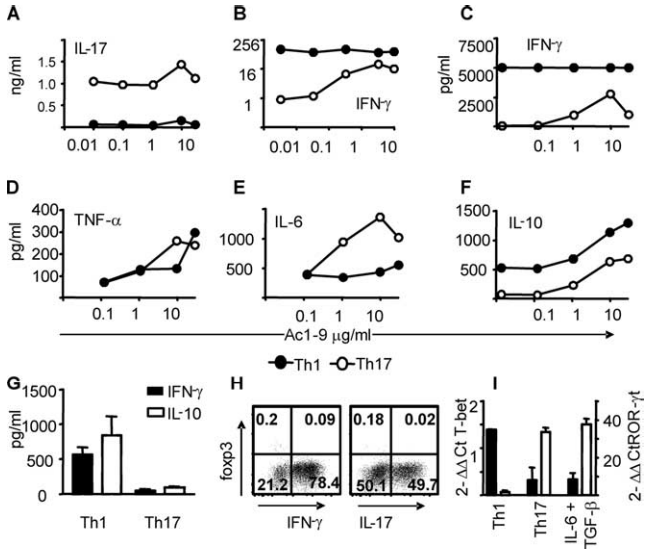

FIGURE 4. Th1-polarized cells are phenotypically stable. *A–F,* Th1-polarized (closed circles) and Th17-polarized (open circles) Tg4 cells were washed extensively and restimulated with graded concentrations of Ac1–9. Cytokine production after 72 h of culture was assessed by ELISA (*A* and *B*) or cytometric bead array (*C–F*). *G*, IFN-γ and IL-10 production by polyclonal Th1- and Th17-polarized cells derived from pMOG-primed mice as determined by ELISA. *H*, Signature cytokine production vs Foxp3 expression in populations of Th1 (*left panel*) and Th17 (*right panel*) Tg4 cells sampled on the day of transfer. *I*, Expression of T-bet (filled bars) and ROR-γt (open bars) at 72 h (expressed as fold induction over cells stimulated under neutral conditions).

disease initiation by transfer into lymphopenic B10.PL × RAG2$^{-/-}$ hosts (Fig. 3, *D–F*). Th17 preparations could induce disease in RAG2$^{-/-}$ recipients (Fig. 3*E*). However, the disease induced with Th1 effectors was always more rapid, with higher mortality. Using the Th1 passive transfer system, we have recently reported that host-derived CD4$^+$Foxp3$^+$ cells are recruited to the inflamed CNS and play a key role in recovery from EAE (9). The absence of these cells in lymphopenic hosts therefore is probably an important factor contributing to this high mortality. Importantly, cells taken from the diseased CNS after transfer of Th17-polarized populations clearly showed the presence of IFN-γ-producing cells. In fact, as illustrated in Fig. 3*F*, IFN-γ-producing cells were found to markedly outnumber IL-17-producing cells in the CNS of these mice.

Signature cytokine production is more stable in Th1 than in Th17 populations

We assessed the stability of in vitro primed Th1 and Th17 populations following restimulation in the absence of exogenous polarizing cytokines. Both populations continued to produce their signature cytokine, but not the opposing cytokine, in the absence of secondary Ag stimulation (Fig. 4, *A* and *B*). We never observed IL-17 production by Th1 preparations, even at high Ac1–9 concentrations (Fig. 4*A*). In contrast high-dose Ac1–9 could provoke IFN-γ production by Th17 preparations (Fig. 4, *B* and *C*). Ag-induced TNF-α production was equivalent in Th1- and Th17-polarized cells (Fig. 4*D*), whereas the latter produced greater amounts of IL-6 (Fig. 4*E*).

Th17-polarized cells do not show increased IL-10 production or Foxp3 expression

It has recently been reported that, in addition to IL-17 production, TGF-β and IL-6 promote IL-10 production, which limits the pathogenic activity of T effectors (and may even confer suppressive activity), whereas IL-23 promotes the production of IL-17 alone, conferring pathogenicity (12). We consistently included IL-23 in our Th17-polarizing conditions alongside TGF-β and IL-6 to maximize pathogenic potential. Furthermore, upon restimulation, Th1-polarized Tg4 populations always produced higher levels of IL-10 than their Th17-polarized counterparts (Fig. 4*F*). Similarly, Th1-polarized populations from pMOG-primed mice produced IL-10, whereas this was negligible in Th17 preparations (Fig. 4*G*). Therefore IL-10 production by Th17-polarized cells does not account for their lack of pathogenicity in this instance. Our Th17 preparations were uniformly Foxp3-negative (Fig. 4*H*). Therefore, we have no evidence for a regulatory component that would inhibit EAE upon the transfer of Th17 preparations. This conclusion is supported by the fact that their inclusion did not suppress the pathogenic activity of Th1 populations (Fig. 1*B*). Populations polarized using only IL-6 and TGF-β did not significantly attenuate disease induced by Th1 effectors (Fig. 1*C*). Thus, our Th1-polarized populations were not sensitive to the suppressive activity of such cells, as reported by McGeachy et al. using IL-23-conditioned pathogenic populations (12). Expression of the lineage-specific transcription factors T-bet and ROR-γt in our pretransfer populations (following primary polarization) showed elevated T-bet expression in Th1 and elevated ROR-γt expression in Th17 preparations, respectively (Fig. 4*I*).

Discussion

Our data provide a picture of Th1 effectors preferentially infiltrating the noninflamed CNS to initiate inflammation that facilitates the recruitment of Th17 cells. These data are clearly at odds with the recent paradigm shift that has led to the role of Th1 cells in EAE being questioned. This has been based largely on active disease models using CFA immunization in gene knockout mice, in which the effects can be complex. For example, exaggerated EAE in IL-12(p35)$^{-/-}$ mice (3) is complicated by their lack of IL-35, which has a reported role in regulatory T cell function (13). To get a definitive picture of their comparative pathogenic potential, it is imperative to transfer disease with Th1 or Th17 cells, avoiding complications that might arise from the use of CFA in the host, and also to use populations that are as devoid of the opposite population as possible. The literature lacks clear evidence for a pure Th17 population, totally deficient in IFN-γ production, having any capacity to initiate CNS autoimmune disease. Previous studies (4) used IL-23-expanded, IL-17-producing preparations that clearly included contaminating IFN-γ$^+$ cells (4, 12). Using such mixed donor populations, the contribution of IFN-γ- and IL-17-producing cells could not be determined. By using traceable populations with truly distinct cytokine secretion profiles, we have been able to dissect the comparative migratory potential and functional stability of Th1 vs Th17 cells with greater exactitude than previously achieved.

Three recent reports of actively induced EAE show that the majority of IL-17-producing CD4$^+$ cells in the CNS also produce IFN-γ (10, 14, 15). We also see these cells after culture with IL-23 alone (Fig. 1*A*), but not after culture with IL-23, IL-6, and TGF-β. However, whenever we saw disease after Th17 transfer, IFN-γ-producing cells were always over-represented in the CNS compared with their frequency at the time of transfer (Fig. 3). The ability of Th17-polarized populations to

gain IFN-γ-producing capacity upon subsequent antigenic stimulation (Fig. 4) may lead to the organ-specific enrichment of IFN-γ^+ and IFN-γ^+IL-17^+ cells in the CNS as has been seen following the transfer of Th17-polarized populations (12). A recent study reporting a pathogenic role for Th17-polarized populations in autoimmune disease of the eye also indicated that their ability to produce IL-17 waned with continued stimulation (16). Consistent with our data, that study showed the induction of disease by a Th1 cell line in the complete absence of IL-17 production (16). Clearly, there is a need for better understanding of the stability of the Th17 phenotype in terms of the production of IL-17 vs other cytokines thought to be more associated with a Th1 response. It may be that either cell can cause pathology under the appropriate conditions and that this may not require the production of the Th1 or Th17 signature cytokines. For example, both cell types can produce significant amounts of TNF-α (Fig. 4). Our data indicate that Th1 cells enter the CNS and modify conditions therein, making it both attractive and accessible to Th17 cells. Understanding the basis for the differential migratory potential and cytokine stability of these polarized populations might allow the development of therapeutic strategies to modulate their distribution and function in vivo.

Disclosures

The authors have no financial conflict of interest.

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